STUDIES ON THE MOVEMENT OF CALCIUM ACROSS THE INNER MITOCHONDRIAL MEMBRANE

by

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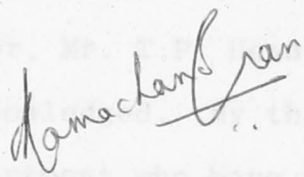
STATEMENT

This thesis describes the results of a study conducted in the Department of Biochemistry, Faculty of Science at the Australian National University, Canberra, under the supervision of Dr. F.L. Bygrave.



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SUMMARY

This thesis examines aspects of the movement of Ca^{2+} across the inner mitochondrial membrane in relation to (1) the role of permeant anions (2) the role of the protonmotive force. As well (3) efflux of the ion from the organelle was investigated.

(1) The role of permeant anions was studied first with tributyltin. The Cl^-/OH^- exchange mediated by this compound in a Cl^--containing medium is shown to be as effective in promoting Ca^{2+} transport as other permeant anions like Pi or acetate. Secondly the role of endogeneous Pi on Ca^{2+} movements was studied using sulphydryl group reagents which inhibit Pi movements. It is shown that mitochondria have a limited capacity to accumulate Ca^{2+} and that the ion is released from the organelle in the presence of these reagents. It is shown also that the entry of Ca^{2+} into mitochondria is fully charge-compensated by the ejection of two H^+.

(2) The role of energy in Ca^{2+} transport was studied by altering the components of the protonmotive force with permeant anions and uncouplers of oxidative phosphorylation as well as by varying the pH of the incubation medium. Ca^{2+} movements are shown to be electrophoretic. The driving force (membrane potential) appears not to limit the initial rate of Ca^{2+} transport. It is shown that an incubation medium of acidic pH decreases influx and increases efflux whereas that of an alkaline pH enhances influx and decreases efflux.

(3) The possible reversal of the Ca^{2+} influx carrier was examined. It is shown that EGTA induces an efflux of Ca^{2+}. This occurs probably by reversal of the Ca^{2+} influx carrier. The properties of this Ruthenium Red-sensitive system are compared with those of the Ruthenium Red-insensitive Ca^{2+} efflux system(s).

PUBLICATIONS

PUBLISHED PAPERS:

1. On the mechanism by which inorganic phosphate stimulates mitochondrial calcium transport
   Bygrave, F.L., Ramachandran, C. & Smith, R.L.
   (1977) FEBS Lett. 83, 155-158.

2. The interaction of tributyltin with mitochondrial calcium transport system of rat liver.
   Bygrave, F.L., Ramachandran, C. & Robertson, R.N.
   (1978) Arch. Biochem. Biophys. 188, 301-307.

3. Calcium-ion cycling in rat liver mitochondria.
   Ramachandran, C. & Bygrave, F.L. (1978) Biochem. J. 174, 613-620.

4. Submitochondrial location of Ruthenium Red-sensitive calcium-ion transport and evidence for its enrichment in a specific population of rat liver mitochondria.
   Bygrave, F.L., Heaney, T.P. & Ramachandran, C.
   (1978) Biochem. J. 174, 1011-1019.

SUBMITTED PUBLICATIONS:

1. The influence of the proton concentration on calcium-ion fluxes across the inner mitochondrial membrane of rat liver.
   Ramachandran, C. & Bygrave, F.L.

2. Ruthenium Red-sensitive and Ruthenium Red-insensitive release of calcium by mitochondria isolated from rat liver and rat heart.

   Ramachandran, C. & Bygrave, F.L.

DEDICATION

DEDICATED TO MY PARENTS

ABBREVIATIONS

CCCP	carbonylcyanide-m-chlorophenylhydrazone
FCCP	carbonylcyanide-p-trifluromethoxyphenylhydrazone
DNP	2,4 -dinitrophenol
EGTA	ethyleneglycol-bis-(β-amino ethyl ether) N,N'-tetracetic acid
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Butyl-PBD	2-(4-ter-Butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole
NTA	nitrilotriacetic acid
NEM	N-ethylmaleimide
PCMBS	p-chloromercuribenzene sulphonate
TBT	tributyltin chloride
state-4	respiration in the absence of Pi acceptor
state-3	respiration in the presence of Pi acceptor
ΔE	membrane potential
ΔpH	pH gradient (when expressed in mV corresponds to $-Z\Delta pH$ where Z at $25^{o}C = 59$)
Δp	protonmotive force

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CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

This thesis examines aspects of the mechanism whereby Ca^{2+} is transported across the inner membrane of mitochondria isolated from rat liver. Since a large body of evidence indicates that mitochondrial Ca^{2+} transport system(s) may play a role in the regulation of cell Ca^{2+} in a range of tissues, this section begins with a summary of the role of Ca^{2+} in the cell and its subcellular distribution. This is followed by a brief, general description of mitochondria and a detailed discussion of the known properties and possible function(s) of the movements of Ca^{2+} across the inner mitochondrial membrane.

THE ROLE OF CALCIUM IN WHOLE BODY AND TISSUE FUNCTION

An adult human body contains 1 to 1.5kg of calcium of which about 98% is located in the skeleton. The regulation of body Ca^{2+} by parathyroid hormone, calcitonin and the metabolites of vitamin D through effects on the absorption of the ion in the intestine, secretion and reabsorption in renal tubules, and deposition and resorption in the skeleton is fairly well established (see for example MacIntyre et al., 1978; Russell, 1978). The exchangeable pool of Ca^{2+} represents less than 1% of the total body Ca^{2+}; about half of it lies outside the skeleton (Carafoli, 1977; Russell, 1978).

The regulation of Ca^{2+} homeostasis in tissues other than bone has been the subject of intense research in the past decade. The concept that Ca^{2+} might play a fundamental role in the regulation of many aspects of cell metabolism appears to be gaining acceptance

(Bygrave, 1967; Rasmussen, 1970; Baker, 1976; Rasmussen et al., 1976; Mikkelsen, 1978). Intracellular Ca^{2+} is thought to act as a mediator in the action of a variety of hormones (Rasmussen, 1970; Rasmussen et al., 1976; Rasmussen & Goodman, 1977; Exton, 1979) and is involved in the activation of contractile and motile systems (see for example Gergely, 1977; Hitchcock, 1977), and of a range of endocrine and exocrine cells and organs concerned with secretion (Douglas, 1974; Carafoli, 1974). It is important also in intracellular communication (Lowenstein & Rose, 1978), and in the activities of a range of regulatory enzymes.

THE SUBCELLULAR DISTRIBUTION AND REGULATION OF CALCIUM

Studies on the fluxes and distribution of Ca^{2+} in rat liver cells have provided evidence for the presence of both extracellular and intracellular pools of the ion (Borle, 1973, 1975; Claret-Berthon et al., 1977). Extracellular pools are exchangeable and represent the bulk of the total Ca^{2+}; this in turn contains both slow- and fast-exchanging components. Internal pools include exchangeable pools within intracellular organelles like mitochondria, the endoplasmic reticulum, the nucleus and possibly the Golgi apparatus as well as the non-exchangeable pools of mitochondria (Claret-Berthon et al., 1977). A wide range of oxyanions are able to bind Ca^{2+} with high specificity (Williams, 1970). The total extracellular Ca^{2+} is about 2.5 mM approx. 40% of which is ionized. The total intracellular Ca^{2+} is in general in the region of 0.2-10 mM (Carafoli & Crompton, 1978) and only a very small amount of the

total Ca^{2+} is ionized (Rasmussen, 1970; Baker, 1976). It is the ionized form that is the 'active' species. Indirect estimates of cytosolic ionic Ca^{2+} appear to be less than 1 μM (Rasmussen, 1970; Gomperts, 1976) which has been confirmed recently by direct measurement in certain cells (Brinley et al., 1977). Consequently a large gradient of ionic Ca^{2+} exists across the plasma membrane, higher outside the cell than inside. This leads to a passive entry of Ca^{2+} into cells down the concentration gradient. In order to prevent the cell from becoming overloaded with Ca^{2+} especially in the case of tissues other than bone, compensatory mechanisms must be present to maintain Ca^{2+} homeostasis against such a gradient. These mechanisms are thought to be located in the plasma membrane, endoplasmic reticulum and mitochondria.

Ca^{2+} transport across the plasma membrane of various tissues appears not to have a common mechanism. Hormone-sensitive binding of Ca^{2+} to plasma membrane has been demonstrated but its relation to the transmembrane movement of Ca^{2+} is uncertain (Bygrave, 1978b). The transport of Ca^{2+} across the erythrocyte plasma membrane using calcium-stimulated ATPase is well documented (Schatzmann & Burgin, 1978), but evidence for the existence of such a system in liver cell membranes is conflicting (Bygrave, 1978b). Other mechanisms like the Na^{+}/Ca^{2+} exchange which has been characterized in nervous tissue have been

suggested to operate in non-excitable tissues (Carafoli & Crompton, 1978). Conclusive proof for the movement of Ca^{2+} across the plasma membrane of liver is still lacking, though various hormones have been shown to induce an efflux of Ca^{2+} from isolated liver cells (Chen et al., 1978).

Microsomes (fragments of endoplasmic reticulum) isolated from a range of tissues have been shown to accumulate Ca^{2+} in an energy-dependent manner (Moore et al., 1975; Bygrave, 1978c; Bygrave & Tranter, 1978). The accumulation process shows a very high affinity for Ca^{2+}, saturability, an absolute requirement of ATP and the ability of weak acids to enhance the rate of transport (Bygrave, 1978c). The properties in some respects are similar to those of the mitochondrial system. The best characterized microsomal Ca^{2+} transport system is that from muscle. The magnesium ATPase complex of muscle has been isolated and successfully reconstituted in artificial vesicles (Tada et al., 1978; Inesi, 1979).

Ca^{2+} transport by mitochondria has been studied in detail in a variety of tissues and will be discussed presently. First some general features about mitochondria will be described.

MITOCHONDRIA

General Features

Cytologists between 1850-1890 observed many granular elements and inclusions in the cytoplasm of the cell. In 1898 Benda (quoted by Lehninger, 1964) used crystal violet as a stain for mitochondria and coined the name

mitochondria from the Greek 'mitos', a thread and 'chondros', a grain. Earlier, cytologists believed that the organelle might have genetic importance in the cell. Kingsbury (1912, see Lehninger, 1964) raised the possibility of these organelles as sites of cellular oxidation and Warburg (1913, see Lehninger, 1964) found respiration associated with these granular insoluble elements of the cell. A variety of workers attempted to isolate mitochondria from broken cell dispersions by differential centriguation (Hogeboom et al., 1948). The method for isolating mitochondria has since been greatly refined mainly due to the painstaking efforts of DeDuve (1975).

Mitochondria are present in the cytoplasm of all eukaryotic cells. The number of mitochondria per cell appears to be relatively constant and characteristic for any given cell type (Lehninger, 1964; Munn, 1974; Whittaker & Danks, 1978). Mitochondria appear to be located near structures that require ATP and make up a relatively large fraction of the cytoplasmic volume; for example about 20% in liver cells and about 50% in heart cells (Lehninger, 1975). Mitochondrial shape can vary from spherical as in brown fat cells, to complex irregular structures as in yeast cells (Munn, 1974). Liver cell mitochondria are cylindrical-shaped being about 2 μ long and about 1 μ wide in intact cells (Lehninger, 1975).

The most salient feature of their structure is the existence of a complex membrane system. It consists of

an outer membrane and a particularly highly-structured inner membrane. The outer membrane is generally smooth, somewhat elastic, and surrounds the mitochondrion like a sac. The inner membrane has a number of invaginations or folds called cristae which penetrate deeply into the intramitochondrial matrix or inner compartment. The main advantage of such a structure appears to be the large increase in surface area. The number of cristae vary with the degree of metabolic activity of the cell. For example mitochondria from highly active tissue like heart and flight-muscle have more cristae than do those from liver. The cristae can undergo changes in configuration depending on the metabolic state of mitochondria (Hackenbrock, 1966). The energy-transducing machinery is located in the cristae (Lehninger, 1964; Green & Baum, 1970; Harmon et al., 1974; Munn, 1974; Racker, 1976; Whittaker & Danks, 1978). In addition the inner membrane contains a large number of carriers for the transport of metabolites (Klingenberg, 1970; LaNoue & Schoolwerth, 1979; Scarpa, 1979). These carriers will be described in detail later.

The space bound by the inner membrane is a gel-like phase called the matrix. The matrix contains large numbers of enzymes, metabolites, ions and the entire protein-synthesizing machinery as well as a unique circular DNA. The space located between the inner and

outer membrane contains some soluble enzymes e.g. adenylate kinase.

In most biological membranes including those of mitochondria, phospholipids appear to be arranged in a bilayer with the polar head groups at the two surfaces and the membrane protein complexes embedded in the bilayer (Singer & Nicolson, 1972; Singer, 1974). These proteins are divided into peripheral (extrinsic) proteins which are largely located in contact with the aqueous sides of the membrane and integral (intrinsic) proteins which are thought to make substantial contact with the hydrophobic region of the membrane (Singer, 1974; DePierre & Ernster, 1977). It is becoming clear that most of the proteins and phospholipids are asymmetrically distributed on the plane of the membrane (Harmon et al., 1974; Singer, 1974; Racker, 1976; DePierre & Ernster, 1977; Nilsson & Dallner, 1977).

From a functional point of view it is the inner membrane of mitochondria that is important and hence we will deal with it hereafter. It has about 80-95% of the membrane-bound mitochondrial proteins and over 90% of the total mitochondrial lipids, most of which are phospholipids. The inner membrane has 75% protein and 25% lipids; 60-70% of the proteins are intrinsic and 90% of the lipids are phospholipids. Cardiolipin, a phospholipid unique to mitochondria, is located in the inner membrane (Colbeau et al., 1971; Harmon et al., 1974;

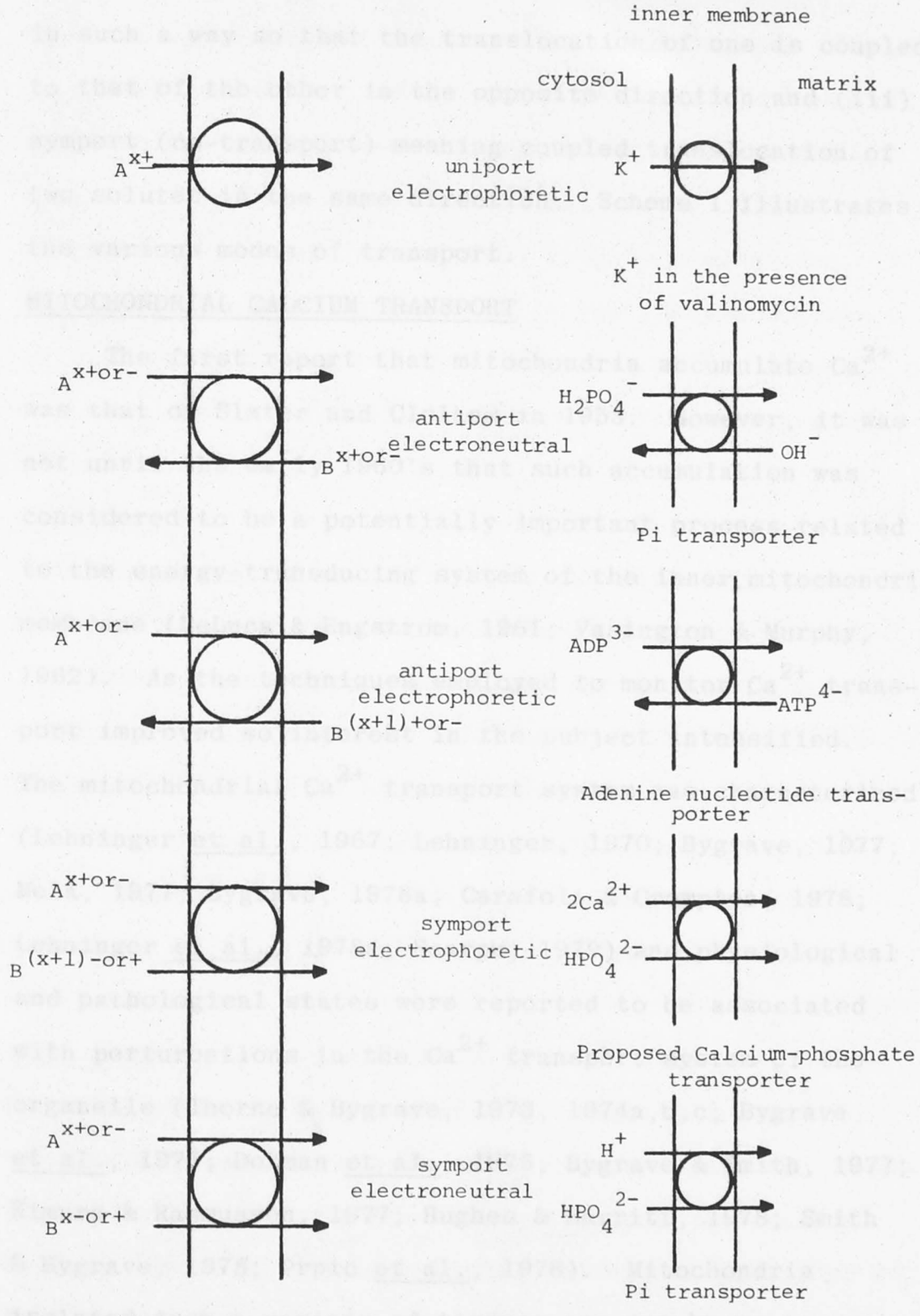
DePierre & Ernster, 1977).

Biogenesis of Mitochondria

It is well established that the mitochondrion possesses a unique species of DNA and RNA and is capable of translation using its own ribosomes (Borst, 1972; Whittaker & Danks, 1978). The synthesis of mitochondria results from a close cooperation of two genetic systems; the nucleocytoplasmic system involving the nuclear DNA and the protein synthesizing apparatus of the cytoplasm, and the mitochondrial system consisting of mitochondrial DNA and the transcription and translation machinery located in the mitochondria (Schatz & Mason, 1974; Schatz, 1979). The two systems are physically separated; they do not share any components and the genomes have no base sequences in common. The intriguing but yet unanswered question is how such different systems coordinate to form functional mitochondria (Schatz, 1979). The cytoplasmic synthesizing system accounts for most of the proteins of the mitochondria (85-95%). These include the proteins of the matrix, outer membrane, most of the inner membrane and those involved in mitochondrial protein synthesis. The proteins formed by the mitochondrial ribosomes are apparently found exclusively in the inner membrane. Though they constitute only 5-15% of the mitochondrial mass they are indispensable for the assembly of a functional mitochondrion (Borst, 1972; Schatz & Mason, 1974; Freedman & Chan, 1978; Schatz, 1979).

Transport of Metabolites Across the Inner Mitochondrial Membrane

The outer membrane has been shown to be freely permeable to a wide range of molecules both charged and uncharged, of molecular weight up to several thousand (Ernster & Kuylenstierna, 1970; Klingenberg, 1970). By contrast, the inner membrane is in general impermeable to all metabolites except H_2O, O_2 and CO_2. Mitochondria are osmotically active behaving as a perfect osmometers (Ernster & Kuylenstierna, 1970). The translocation of a metabolite across the inner membrane depends on its nature, specifically whether it is charged or uncharged, lipophilic or hydrophilic (Hall & Baker, 1977; LaNoue & Schoolwerth, 1979). In general lipophilic substances enter the mitochondria by passive diffusion, whereas the translocation of charged molecules requires the presence of a carrier. These carriers are specific and catalyse the translocation of metabolites across the hydrophobic core of the membrane (Bangham, 1972; Whittaker & Danks, 1978; LaNoue & Schoolwerth, 1979; Scarpa, 1979). The nature of transport can be (i) electrogenic, i.e. it causes a perturbation in the charge difference across the membrane, or (ii) electroneutral, i.e. it causes no change in the charge difference across the membrane. The mechanism of transport can be (i) uniport, i.e. translocation of a single metabolite by the carrier (ii) antiport (counter-transport) meaning transport of two solutes



Scheme 1 Transport of metabolites across the membrane

in such a way so that the translocation of one is coupled to that of the other in the opposite direction and (iii) symport (co-transport) meaning coupled translocation of two solutes in the same direction. Scheme 1 illustrates the various modes of transport.

MITOCHONDRIAL CALCIUM TRANSPORT

The first report that mitochondria accumulate Ca^{2+} was that of Slater and Cleland in 1953. However, it was not until the early 1960's that such accumulation was considered to be a potentially important process related to the energy-transducing system of the inner mitochondrial membrane (DeLuca & Engstrom, 1961; Vasington & Murphy, 1962). As the techniques employed to monitor Ca^{2+} transport improved so interest in the subject intensified. The mitochondrial Ca^{2+} transport system was characterized (Lehninger et al., 1967; Lehninger, 1970; Bygrave, 1977; Mela, 1977; Bygrave, 1978a; Carafoli & Crompton, 1978; Lehninger et al., 1978a; Scarpa, 1979) and physiological and pathological states were reported to be associated with perturbations in the Ca^{2+} transport system of the organelle (Thorne & Bygrave, 1973, 1974a,b,c; Bygrave et al., 1975; Dorman et al., 1975; Bygrave & Smith, 1977; Kimura & Rasmussen, 1977; Hughes & Barritt, 1978; Smith & Bygrave, 1978; Prpic et al., 1978). Mitochondria isolated from a variety of tissues are now known to accumulate large amounts of Ca^{2+} (Lehninger, 1970; Bygrave, 1977; Mela, 1977; Carafoli & Crompton, 1978)

in an energy-dependent manner. The physiological role of mitochondrial Ca^{2+} accumulation is largely unknown in non-bone forming tissues, though it has been suggested that it might play a role in the regulation of cell Ca^{2+} (Bygrave, 1967; Lehninger, 1970) thereby controlling metabolic events sensitive to this ion (Meli & Bygrave, 1972; Roberts & Bygrave, 1973; Bygrave, 1978a; Carafoli & Crompton, 1978). Details of the Ca^{2+} transport system are now described.

Ca^{2+} Carrier

The transport of Ca^{2+} across the inner mitochondrial membrane is thought to be mediated by a carrier (Mela, 1967,1969), the evidence for which arises mainly from kinetic studies (Bygrave, 1977; Mela, 1977; Carafoli & Crompton, 1978). The site of location of the carrier is the inner membrane (Klingenberg, 1970; Pedersen & Coty, 1972; Bygrave, 1977). The kinetic evidence for the existence of a carrier arises mainly from the following considerations.

High affinity: The mitochondrial Ca^{2+} transport system has been shown to have a very high affinity for Ca^{2+} (Km 2-4 μM) by the use of techniques which measured only the transported Ca^{2+} (i.e. that not accessible to EGTA) (Reed & Bygrave, 1975a,b). Though higher values, of the order of 50 μM (Scarpa & Graziotti, 1973; Vinogradov & Scarpa, 1973) have been reported, such high values are probably due to the presence of Mg^{2+} which is now known to be a competetive inhibitor of mitochondrial Ca^{2+} transport (Hutson et al., 1976;

Åkerman, 1977; Hutson, 1977). By comparison, the affinity of the carrier for Ca^{2+} is higher than that of ADP for phosphorylation (Lehninger et al., 1967; Jacobus et al., 1975; Bygrave, 1977).

Sigmoidicity and Saturation kinetics: The initial velocity versus substrate concentration plot shows clear evidence for sigmoidicity with saturation kinetics (Bygrave et al., 1971; Spencer & Bygrave, 1973; Scarpa & Graziotti, 1973; Vinogradov & Scarpa, 1973; Scarpa, 1974; Reed & Bygrave, 1975b; Åkerman, 1977). The Hill plot yields a Hill coefficient of approximately 2 which provides strong evidence for positive co-operativity. This suggests that at least two sites may be involved in the interaction of Ca^{2+} with the carrier (Bygrave, 1977; see also Tew, 1977).

Inhibitors: Lanthanides and Ruthenium Red are known to inhibit mitochondrial Ca^{2+} transport.

Lanthanides bind strongly to Ca^{2+}-binding sites in general (Williams, 1970, 1976) and are known to competitively inhibit mitochondrial Ca^{2+} transport (Mela, 1967, 1969; Reed & Bygrave, 1974a); the Ki is about 2×10^{-8}M. The number of lanthanide-binding sites is approximately 1 pmole/mg of protein. The inhibition by lanthanides is transient (Reed & Bygrave, 1974a) presumably due to its transport into the mitochondrion (Reed & Bygrave, 1974a,c).

Ruthenium Red is a mucopolysaccharide stain and has been shown to specifically inhibit mitochondrial Ca^{2+}

transport (Moore, 1971; Vasington et al., 1972; Reed & Bygrave, 1974a) non-competitively (Reed & Bygrave, 1974a) at low concentrations. The Ki is 3×10^{-8}M and the number of the Ruthenium Red-binding sites is about 80 pmoles/mg of mitochondrial protein (Reed & Bygrave, 197a). The specificity of Ruthenium Red to inhibit mitochondrial Ca^{2+} transport has led to its use to distinguish between mitochondrial (Ruthenium Red-sensitive) and non-mitochondrial (Ruthenium Red-insensitive) Ca^{2+}-transport systems in studies of cell Ca^{2+} (Ash & Bygrave, 1977; Bygrave & Tranter, 1978).

Other compounds like hexamine cobalti-chloride, another mucopolysaccharide stain (Tashmukhamedov et al., 1972) and (+)-α-(N-1-phenethyl) urea (Davidoff et al., 1976) have also been shown to inhibit mitochondrial Ca^{2+} transport.

Binding of Ca^{2+} by Mitochondria

If a carrier is involved in the translocation of Ca^{2+} across the inner mitochondrial membrane, the primary event in the translocation would be the binding of Ca^{2+} to the proposed carrier (Bygrave, 1977). Attempts to measure carrier-specific binding have revealed two classes of binding sites, one of high and one of low affinity (Lehninger, 1969; Reynafarje & Lehninger, 1969; Carafoli & Lehninger, 1971).

High affinity binding sites were very similar in properties to the Ca^{2+} transport system and were equated to the carrier-specific binding of Ca^{2+} (Lehninger, 1969;

Reynafarje & Lehninger, 1969). The number of Ca^{2+} binding sites reported (about 1nmole/mg of mitochondrial protein) was at least an order of magnitude higher than that predicted by inhibitor studies. In addition such binding was reported to be sensitive to uncouplers which is rather surprising as the carrier is known to operate independently of metabolism (Selwyn et al., 1970a). In 1974 three groups independently reported that the high affinity sites measured, reflected the transport of a limited amount of Ca^{2+} (Åkerman et al., 1974; Reed & Bygrave, 1974b; Southard & Green, 1974) utilizing the energy reserves. The technical difficulties associated with the measurement of such sites were discussed by Reed & Bygrave, (1974b). It is interesting to note that the inhibition profile of respiration-dependent binding or transport of Ca^{2+} by members of the lanthanide series shows a different pattern than that of respiration-independent binding suggesting that the former binding sites may involve sites different to those from the latter (Tew, 1977; Lehninger et al., 1978a).

Low affinity binding involves nonspecific binding sites located on both the outer and the inner membrane (Chappell et al., 1963; Rossi et al., 1967; Scarpa & Azzi, 1968; Reynafarje & Lehninger, 1969; Scarpa & Azzone, 1970; Vainio et al., 1970; Reed & Bygrave, 1974b). These sites were thought to be polar head groups of phospholipids based on their sensitivity to Na^{+}, K^{+} and local

anaesthetics (Scarpa & Azzone, 1970; Vainio et al., 1970; Reed & Bygrave, 1974b). The number of sites are about 15 nmoles/mg of mitochondrial protein (Reed & Bygrave, 1974b) and the function of such sites is largely unknown (Bygrave, 1977).

Ca^{2+}-binding Carrier Proteins

Mitochondrial glycoproteins capable of binding Ca^{2+} have been isolated by osmotic shock (Lehninger, 1971; Gomez-Puyou et al., 1972; Sottocasa et al., 1972,1977). The properties of the isolated proteins, especially the binding affinity for Ca^{2+} and sensitivity to Ruthenium Red (Lehninger, 1971; Gomez-Puyou et al., 1972; Sottocasa et al., 1972) has led to the suggestion of its involvement in Ca^{2+} transport. The reconstitution of the Ca^{2+} transport system in artificial vesicles using the isolated protein has not been successful. The protein has been shown to increase the electrical conductance of an artificial membrane formed from lecithin (black film) in the presence of Ca^{2+} which was sensitive to Ruthenium Red (Carafoli & Sottocasa, 1974; Prestipino et al., 1974; Carafoli, 1976). It also partially restore the Ca^{2+} transporting ability of mitochondria devoid of the glycoprotein (Sandri et al., 1979). The protein was thought to behave as a surface receptor(s) or recognition site(s) for Ca^{2+} and may function in conjunction with a transmembrane carrier (Sottocasa et al., 1977).

More convincing evidence for the involvement of

the glycoprotein in mitochondrial Ca^{2+} transport was provided by the demonstration that low concentrations of antibodies raised against the glycoprotein could specifically inhibit mitochondrial Ca^{2+} transport (Panfili et al., 1976). Recently a low molecular weight protein was also isolated from mitochondria, and its ability to bind divalent cations with high affinity led to the suggestion that it was involved in mitochondrial Ca^{2+} transport (Jeng et al., 1978). Peptides having ionophorous activities have been isolated from mitochondria and have been thought to play a role in mitochondrial ion transport (Green & Blondin, 1977) but the possible involvement of these peptides remain unknown.

THE ROLE OF ENERGY IN CALCIUM TRANSPORT

Energy-dependent Ca^{2+} Transport

It is well known that mitochondria accumulate Ca^{2+} in an energy-dependent manner (Lehninger, 1970; Bygrave, 1977; Mela, 1977; Bygrave, 1978a,b; Carafoli & Crompton, 1978; Lehninger et al., 1978a). Energy may be provided either by respiration i.e. the oxidation of various substrates by the respiratory chain or by ATPase activity i.e. hydrolysis of ATP by reversal of the ATP synthetase.

The fundamental requirement of energy for Ca^{2+} transport provided either by electron transport or ATP hydrolysis, is the charge separation leading to the extrusion of protons on the outside and hydroxyl ions on the matrix side of the membrane as envisaged by Mitchell

(1961, 1966, 1967, 1968, 1977a,b). This charge separation, originally proposed by Lundegårdh (quoted in Robertson, 1968) and enunciated in detail by Mitchell, in his formulation of the chemiosmotic hypothesis, leads to the generation of the protonmotive force (Mitchell, 1966, 1967, 1968; Mitchell & Moyle, 1969a,b; Rottenberg, 1975). The magnitude of the protonmotive force is of the order of 230 mV in rat liver mitochondria (Mitchell & Moyle, 1969a; Nicholls, 1974; Rottenberg, 1975). The protonmotive force is now known to contain two components, the membrane potential and the pH gradient.

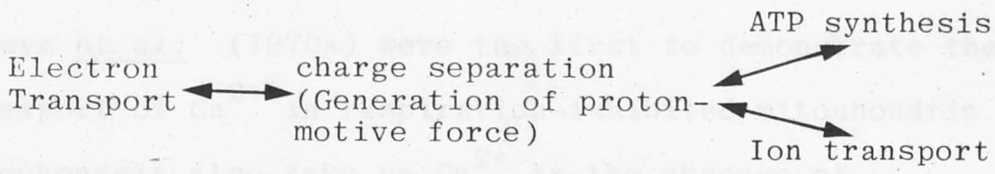
The membrane potential is thought to arise from the differences between the surface potentials on the two sides of the membrane. These potentials are produced by the ionization of molecules at the membrane surfaces and in the surrounding medium (Mitchell & Moyle, 1969a; Ohki, 1973; Rottenberg, 1975; Mitchell, 1977b). It is likely that both the surface potential and the diffusion potential contribute to the membrane potential.

The pH gradient is the chemical or osmotic component and is thought to arise because of the difference in the proton concentration between the two sides of the membrane (Mitchell & Moyle, 1969a; Mitchell, 1977b).

In mitochondria and in certain microorganisms, the membrane potential is the major component of the proton-motive force under physiological conditions, whereas in some microorganisms which grow in very acidic environments the pH gradient may be the major component of the proton-

motive force (Mitchell, 1970).

Mitchell has suggested that it is the protonmotive force that causes coupling between respiration and phosphorylation. He also postulated the existence, in the energy transducing membrane, of a series of porters (carriers) which catalyze the movement of various substrates and ions in response to the protonmotive force (Mitchell & Moyle, 1969a,b; Klingenberg, 1970; Mitchell, 1977b; LaNoue & Schoolwerth, 1979; Scarpa, 1979). In its simplest form the chemiosmotic hypothesis can be represented schematically as follows:-



It is electron transport that causes the charge separation which leads to the generation of a protonmotive force which, in turn, can be used either to drive ATP synthesis or ion movements. The reactions are reversible and ion gradients have been shown to drive ATP synthesis (see for example Ryrie & Blackmore, 1976). There is considerable evidence for the existence of a variety of translocators in the inner mitochondrial membrane (Klingenberg, 1970) and anions are now known to be distributed according to the pH gradient (Klingenberg, 1970).

Although the transport of Ca^{2+} across the inner mitochondrial membrane is thought to be an electrophoretic

process in response to the membrane potential, negative inside, very few direct experiments have tested this possibility (Selwyn et al., 1970a; Lehninger, 1974; Rottenberg & Scarpa, 1974; Heaton & Nicholls, 1976). Indeed, it is only now that much of the information on the electrophoretic nature of Ca^{2+} transport and its relation to the components of the protonmotive force has appeared while this work was in progress (Åkerman, 1978a; Nicholls, 1978a,b).

Energy-independent Ca^{2+} Transport

Mitochondria can also transport Ca^{2+} in an energy-independent manner under respiration-inhibited conditions. Selwyn et al. (1970a) were the first to demonstrate the transport of Ca^{2+} in respiration-inhibited mitochondria. Mitochondria also take up Ca^{2+} in the absence of respiration in exchange for intramitochondrial K^{+} in the presence of valinomycin (Scarpa & Azzone, 1970; Azzone et al., 1977; Åkerman, 1978b) with a stoichiometry of 2 K^{+} ions for one Ca^{2+} ion (Azzone et al., 1977; Åkerman, 1978b). With the use of EGTA to distinguish between the binding and transport of Ca^{2+}, it was conclusively demonstrated that mitochondria, in the absence of respiration and ATP hydrolysis, can transport Ca^{2+} into the EGTA-inaccessible space of mitochondria (Reed & Bygrave, 1974b). The movement of Ca^{2+} in respiration-inhibited mitochondria is thought to be carrier-mediated based on the sensitivity to lanthanides and Ruthenium Red (Selwyn et al., 1970a; Scarpa & Azzone, 1970; Reed

& Bygrave, 1974b).

Mode of Mitochondrial Ca^{2+} Transport

The mode of movement of Ca^{2+} across the inner membrane is largely unknown. The transport process appears to be electrogenic (Selwyn et al., 1970a). Three different electrogenic models have been proposed. They are:
(i) uniport mode in which the transport of Ca^{2+} across the inner membrane is equivalent to the transfer of two positive charges across the membrane (Selwyn et al., 1970a; Lehninger, 1974; Rottenberg & Scarpa, 1974; Heaton & Nicholls, 1976; Azzone et al., 1977)
(ii) antiport mode in which the transport of one Ca^{2+} is coupled to the efflux of one proton (Reed & Bygrave, 1975b) and
(iii) symport mode in which there is an obligatory coupling between the transport of Ca^{2+} and permeant anions in such a way that the transfer of one Ca^{2+} is equivalent to the transfer of one positive charge across the inner membrane (Moyle & Mitchell, 1977a,b,c; see also Reynafarje & Lehninger, 1977). The various mechanisms have been the subject of much debate and the real mode of transport still is not known, though the uniport model is favoured by most workers.

Stoichiometry

Ca^{2+} addition to mitochondria leads to either a stimulation in the rate of oxygen uptake in the presence of respiratory substrates or a burst of ATP hydrolysis in the presence of ATP. The stoichiometry of the process

was studied in detail in a variety of laboratories (for review see Lehninger et al., 1967). Rossi & Lehninger (1964) reported that with respiratory substrates, approximately 2 Ca^{2+} ions are accumulated for every pair of electrons traversing each of the three energy-conserving sites and phosphate was accumulated with a stoichiometry of about 1.7 phosphate ions for two Ca^{2+} ions. Bielawski & Lehninger (1966) found that with ATP as an energy source, for each molecule of ATP hydrolysed about 1.9 molecules of Ca^{2+} were accumulated along with one molecule of phosphate. Ca^{2+} uptake by mitochondria is associated with proton ejection as first reported by Saris (1963) and in the absence of permeant anions one H^+ was ejected for each Ca^{2+} ion accumulated (Lehninger et al., 1967) and the ratio was decreased by the presence of permeant anions. Recently the role played by endogeneous phosphate in reducing the H^+/Ca^{2+} stoichiometry was realized when an inherent stoichiometry of 2 H^+ for each Ca^{2+} was demonstrated (see Lehninger et al., 1978a for a review).

Efflux of Ca^{2+} from Mitochondria

Sordahl (1975) suggested that Ca^{2+} is released from the mitochondria by a route different from that of the influx system. The idea was not accepted until quite recently when the evidence for a separate efflux mechanism has grown considerably (Puskin et al., 1976; Carafoli & Crompton, 1978; Lehninger et al., 1978a; Nicholls, 1978b). From the physiological stand point, if the mitochondrial Ca^{2+} transport has to play a role in the cellular

homeostasis of Ca^{2+}, for which the evidence is mounting, the Ca^{2+} sequestered by the organelle must be available for rapid release. The major evidence for an efflux mechanism is as follows:-

Thermodynamic: Studies on the distribution of Ca^{2+} in the steady-state by using Mn^{2+} as a paramagnetic analogue of Ca^{2+}, have revealed that the distribution of free Mn^{2+} in the steady-state does not obey the Nernst equation (Puskin et al., 1976; Azzone et al., 1977). It was reasoned that an efflux pathway could prevent equilibrium from being attained (Puskin et al., 1976).

Effect of Ruthenium Red: It is well established that Ruthenium Red is a non-competetive inhibitor of Ca^{2+} influx (Bygrave, 1977). The effect of Ruthenium Red on the release of Ca^{2+} from mitochondria appears to vary (Carafoli & Crompton, 1978). In general the release of Ca^{2+} is much less sensitive to Ruthenium Red than influx, again suggesting that the route of release of Ca^{2+} is different from that of influx (Sordahl, 1975; Puskin et al., 1976; Carafoli & Crompton, 1978; Lehninger et al., 1978a).

Kinetic evidence: The recent demonstration that mitochondria precisely regulate the extramitochondrial free Ca^{2+} concentration at around 0.8 μM by altering either the rate of uptake or release provides conclusive evidence for an efflux mechanism (Nicholls, 1978b).

It is known that the interruption of the energy flow leads to the release of Ca^{2+} from mitochondria (Carafoli, 1974: Bygrave, 1977; Mela, 1977; Bygrave, 1978a; Carafoli & Crompton, 1978). The release of Ca^{2+} has a high temperature quotient (Lehninger et al., 1967). In addition a large number of pharmacological agents can cause release of Ca^{2+} from mitochondria (Mela, 1977; Bygrave, 1978a; Carafoli & Crompton, 1978) presumably by interfering with the energy supply of mitochondria (Carafoli & Crompton, 1978). The search for physiological agents causing release of Ca^{2+} led to the identification of compounds like phosphoenol pyruvate, Na^{+}, cyclic AMP and palmitoyl CoA.

Phosphoenol pyruvate: Phosphoenol pyruvate at very low concentrations causes a release of Ca^{2+} in the presence of phosphate (Chudapongse & Haugaard, 1973; Peng et al., 1974; Chudapongse, 1976). Such a release of Ca^{2+} was shown to be due to the exchange of phosphoenol pyruvate with adenine nucleotides (Chudapongse & Haugaard, 1973; Peng et al., 1974; Sul et al., 1976). Recently it was shown that the release of Ca^{2+} induced by phosphoenol pyruvate is due to the uncoupling of mitochondria (Ross et al., 1978).

Sodium ions: Na^{+} has been shown to influence the Ca^{2+} transporting ability of mitochondria isolated from tissues like heart and brain, but not from liver or kidney (Carafoli et al., 1974; Crompton et al., 1976, 1977; Carafoli & Crompton, 1978; Crompton et al., 1978). In the former tissues Na^{+} depresses Ca^{2+} uptake and induces Ca^{2+} release

(Carafoli & Crompton, 1978). Addition of Na^+ to mitochondria preloaded with Ca^{2+} and inhibited with Ruthenium Red (to prevent reuptake of released Ca^{2+}) leads to a rapid efflux of intramitochondrial Ca^{2+} (Crompton et al., 1976,1977,1978). The system is thought to catalyse an exchange between Na^+ and Ca^{2+}, stimulating efflux of Ca^{2+} which is not sensitive to Ruthenium Red (Carafoli & Crompton, 1978) but sensitive to oligomycin (Harris, 1977) and was associated with no damage to mitochondria (Nicholls, 1978a). In heart mitochondria, Na^+-induced efflux of Ca^{2+} has a maximum velocity of about 15 nmoles/mg of mitochondrial protein/min at 25°C. The Km for Na^+ was about 8 mM and the maximal rate of Ca^{2+} efflux was attained by 13 mM Na^+ (Crompton et al., 1976). Na^+-induced Ca^{2+} release has been suggested to play a role in the reguation of cell Ca^{2+} in the affected tissue (Crompton, et al., 1977: Carafoli & Crompton, 1978).

Cyclic AMP: Cyclic AMP was shown to cause a release of Ca^{2+} from rat liver, heart and kidney mitochondria (Borle, 1974), but the inability to reproduce such a finding raises doubt about its significance (Borle, 1976; Scarpa et al., 1976).

Palmitoyl CoA: Palmitoyl CoA induces an efflux of Ca^{2+} from mitochondria which is not sensitive to Ruthenium Red. The release was inhibited by carnitine (Asimakis & Sordahl, 1977). The release occurs, presumably due to release of adenine nucleotides (Asimakis & Sordahl, 1977).

Pyridine nucleotide redox state: Recently it was

discovered that the redox state of pyridine nucleotides are important in the control of mitochondrial Ca^{2+} transport (Lehninger et al., 1978a,b). Reduction of pyridine nucleotides enables the mitochondria to retain Ca^{2+} whereas the oxidation of pyridine nucleotides causes the mitochondria to release Ca^{2+}. These findings were confirmed in our laboratory and extended by showing that only NADPH (and not NADH) was important in controlling Ca^{2+} uptake and release (Prpic & Bygrave, 1980). Such a mechanism has obvious physiological implications as the redox state of the pyridine nucleotides are known to change with changes in metabolic activity. (Fiskum & Lehninger, 1979: Prpic & Bygrave, 1980).

The Ca^{2+}-translocation Cycle

Evidence for a cyclic flux of Ca^{2+} existed as early as 1965, and low concentration of Ca^{2+} were retained by the mitochondria during state 4 respiration not as a result of irreversible sequestration of the ion but rather as a reflection of a dynamic steady-state in which the efflux of Ca^{2+} down the concentration gradient was counter-balanced by energy-linked accumulation of Ca^{2+} (Drahota et al., 1965). Conclusive evidence for Ca^{2+} cycling was provided by Stucki & Ineichen (1974), but the rate of the process was very slow (endogeneous Ca^{2+} cycling of about 4 nmoles/mg of mitochondrial protein at $37^{o}C$). Recently the cyclic movement of Ca^{2+} across the inner mitochondrial membrane has been considered analogous (Bygrave, 1978a; Crompton & Heid, 1978; Nicholls, 1978b; Carafoli, 1979), to the substrate cycling in metabolism

(Newsholme & Crabtree, 1976). Cycling of Ca^{2+} has the advantage of providing efficient feed-back control by increasing the sensitivity of the reactions influenced by the ion (Bygrave, 1978a). Large number of effectors are now known to modulate either the influx or efflux or both components of the Ca^{2+}-translocation cycle including Ca^{2+} itself (Lehninger, 1970; Bygrave, 1977; Mela, 1977; Bygrave, 1978a; Carafoli & Crompton, 1978; Lehninger et al., 1978a), proton donating permeant anions (Selwyn et al., 1970a; Lehninger, 1974; Reed & Bygrave, 1975b), adenine nucleotides (Out et al., 1971; Peng et al., 1974; Sul et al., 1976; Asimakis & Sordahl, 1977), ions like Mg^{2+} (Hutson et al., 1976; Åkerman, 1977; Hutson, 1977), Na^{+} in some tissues (Carafoli et al., 1974; Crompton et al., 1976,1977,1978; Crompton & Heid, 1978) and hormones (Dorman et al., 1975; Kimura & Rasmussen, 1977; Bygrave, 1978b; Hughes & Barritt, 1978; Prpic et al., 1978; Taylor et al., 1980).

Permeant anions: Anions like phosphate are known to influence the influx and efflux components of the Ca^{2+}-translocation cycle (Bygrave, 1978a). Anions can be classified into two categories, permeant anions like phosphate, acetate and thiocyanate, and impermeant anions like chloride and perchlorate (Chappell, 1968; Mitchell & Moyle, 1969b). Among the permeant anions only those that have the potential ability to donate a proton to the matrix such as phosphate and acetate can support Ca^{2+}

accumulation by mitochondria (Lehninger, 1974; Reed & Bygrave, 1975b). The proton-donating permeant anions convert the intramitochondrial alkaline electrochemical gradient of protons into the negative gradient of the transported anion which is thought to act as a pulling force for the Ca^{2+} (Lehninger, 1974). In the absence of proton-donating permeant anions, mitochondria have a limited capacity (about 80 nmoles/mg of mitochondrial protein) to accumulate Ca^{2+} (Lehninger et al., 1967). The presence of proton-donating permeant anions increases both the rate and extent of mitochondrial Ca^{2+} transport (Rossi & Lehninger, 1964; Rasmussen et al., 1965; Chance & Yoshida, 1966; Mela & Chance, 1968). Kinetic studies have revealed that anions enhancing mitochondrial Ca^{2+} transport do so by increasing the Vmax without affecting the Km (Spencer & Bygrave, 1973; Reed & Bygrave, 1975b). Such studies have revealed that the rate-limiting step in mitochondrial Ca^{2+} transport is probably the rate of dissociation of Ca^{2+} from the Ca^{2+} carrier complex on the matrix side (Reed & Bygrave, 1975b). Electron paramagnetic resonance studies have revealed that there is no direct interaction of phosphate with the divalent cation carrier (Case, 1975). Proton-donating permeant anions, in addition to their ability to decrease the matrix pH (Rasmussen et al., 1965; Chance & Mela, 1966; Gear et al., 1967; Nicholls, 1974) also provide a co-ion

for the transported Ca^{2+}. It has been calculated that a fall in matrix pH of about 0.3 units could double the initial rate of transport (Reed & Bygrave, 1975b).

Adenine nucleotides: Ever since the finding that either respiration or ATP hydrolysis could support energy-dependent transport of Ca^{2+} by mitochondria, the role of adenine nucleotides in the transport of the ion has been examined, especially with reference to the control of the process. During massive accumulation of Ca^{2+}, adenine nucleotides have been reported to be taken up (Carafoli et al., 1965) and extra-mitochondrial ATP was thought to enhance the precipitation of calcium phosphate in the matrix (Lehninger, 1970). Low concentrations of extra-mitochondrial Ca^{2+} have been shown to specifically stimulate the translocation of ATP (Spencer & Bygrave, 1972) whereas the transported Ca^{2+} inhibits adenine nucleotide translocase activity (Gomez-Puyou et al., 1979). Retention of Ca^{2+} by mitochondria has been correlated with the mitochondrial adenine nucleotide content under some conditions and/or tight binding of the nucleotide to the translocase (Out et al., 1971; Kimura & Rasmussen, 1977; Prpic et al., 1978).

Magnesium ions: Heart mitochondria are the only organelles known to accumulate large amounts of Mg^{2+} in a respiration-dependent process (Lehninger et al., 1967; Brierley et al., 1970). Although it has been reported that rat liver mitochondria isolated free of

lysosomes transport Mg^{2+} in the presence of ATP, the significance of the finding is questionable because of the high Km and the very slow rates of transport observed (Kun, 1976; Diwan et al., 1979). In spite of its inability to be transported in any appreciable amount, Mg^{2+} has been shown to influence both the influx and efflux components of the Ca^{2+}-translocation cycle. Mg^{2+} decreases the initial rate of Ca^{2+} transport in a competitive manner and hence increases the Km for Ca^{2+} for the mitochondrial Ca^{2+}-transporter (Hutson et al., 1976; Åkerman, 1977; Hutson, 1977). Therefore it was thought that the affinity of mitochondria for Ca^{2+} may be lower than that for ADP in the presence of Mg^{2+} (Hutson, 1977), especially because the cytosol contains large amounts of Mg^{2+} (Lehninger et al., 1967). Mg^{2+} has been reported to change the shape of the kinetic plot from sigmoidal to hyperbolic or to alter the region at which sigmoidicity occurs (Åkerman, 1977). In addition to its ability to inhibit the initial rate of Ca^{2+} transport, Mg^{2+} may stabilize the mitochondrial membrane against the deleterious effect of Ca^{2+} (Haugaard et al., 1969a & b; Harris, 1979; Hunter & Haworth, 1979).

Physiological Regulation of the Ca^{2+}-translocation Cycle

A role for Ca^{2+} as one of the mediators of hormone action has been proposed (Rasmussen, 1970; Rasmussen & Goodman, 1977). Mitochondrial Ca^{2+} transport has been

shown to be altered during development (Bygrave, 1977, 1978a), in a variety of tumour cells (Bygrave, 1976) and was suggested to be a possible target for hormone action (Dorman et al., 1975).

Development: Ca^{2+} transport by mitochondria isolated from the flight-muscle of the blowfly (Lucilia cuprina), has been shown to change with development (Bygrave et al., 1975; Smith & Bygrave, 1978). The transport activity was low before emergence and increased steadily with maximal activity occuring just after emergence; it then decreased slowly (Bygrave et al., 1975). The changes in Ca^{2+} transporting ability did not reflect changes in the activity of energy transduction (Smith & Bygrave, 1978).

Ca^{2+} transport by mitochondria isolated from rat liver has also been shown to change during development of the rat liver (Bygrave, 1977; Bygrave & Ash, 1977). The activity has been shown to be lower before birth and to markedly increase one to two days after birth in the presence of MgATP to reach adult levels (Bygrave & Ash, 1977). MgATP also enhances the retention of Ca^{2+} by foetal mitochondria (Pollak, 1975).

Tumour cell: Mitochondria isolated from Ehrlich ascites tumour cells have been shown to be resistant to the uncoupling action of Ca^{2+} (Reynafarje & Lehninger, 1973; Thorne & Bygrave, 1973; McIntyre & Bygrave, 1974; Thorne & Bygrave, 1974a,b). In these tumour cells Ca^{2+} inhibits

the translocation of adenine nucleotides across the inner mitochondrial membrane (Thorne & Bygrave, 1974b,c, 1975) rather than enhancing it as in rat liver (Spencer & Bygrave, 1972). It is also interesting to note that only a small percentage of the adenine nucleotides of tumour cell mitochondria are exchangeable (Bygrave, 1976) and is thought to be due to a higher endogeneous Ca^{2+} in these mitochondria (Eboli et al., 1979).

Hormones and mitochondrial Ca^{2+} transport: In vivo administration of hormones to rats leads to marked changes in the components of the Ca^{2+}-translocation cycle in the isolated mitochondria (Dorman et al., 1975). Mitochondria isolated after administration of corticoids exhibit lowered initial rates of Ca^{2+} transport and a decrease in the ability to retain accumulated Ca^{2+}. The latter change was thought to be due to a decrease in the ATP content of the mitochondria (Kimura & Rasmussen, 1977). Glucagon administration to whole rats or perfused livers significantly enhanced the ability of subsequently isolated mitochondria to retain Ca^{2+} (Hughes & Barritt, 1978; Prpic et al., 1978). A similar effect was also observed in the perfused system with α-adrenergic agonists (Taylor et al., 1980). These alterations were associated with an increased transmembrane pH gradient, a decreased ability to oxidize NADPH induced by oxaloacetate and an increased mitochondrial adenine nucleotide content. At this stage possible casual relationship between the changes described have not been identified.

Physiological Role of the Mitochondrial Ca^{2+} Transport System:

The physiological role of the mitochondrial Ca^{2+} transport system is largely unknown, though it has been thought to play a role in the regulation of the ionic environment of the cell (Lehninger et al., 1967; Rasmussen, 1970; Lehninger, 1970; Bygrave, 1977; Mela, 1977; Rasmussen & Goodman, 1977; Bygrave, 1978a,b; Carafoli & Crompton, 1978). The most compelling evidence for a physiological role for the transport system stems from: (i) the extremely high affinity of the system for Ca^{2+} (See P 11)

(ii) the in vitro demonstration of physiological agents to cause a release of Ca^{2+} (see P 23).

(iii) direct demonstration of the ability of mitochondria to monitor cytosolic Ca^{2+} in vivo (Rose & Lowenstein, 1975) and

(iv) the susceptibility of the system to dietary control (Bygrave & Smith, 1977), or hormone administration (see P 31) as well as a permanent derangement of the transport system in certain tumour cells (see P 30), and changes in the activity of the system during development (see P 30).

Mitochondria isolated from a wide variety of tissues are known to contain some endogeneous Ca^{2+} (Lehninger et al., 1967). In vivo administration of radioactive Ca^{2+} followed by isolation of mitochondria (with care to minimise redistribution of Ca^{2+}) has revealed that large

amounts of radioactivity are associated with mitochondrial fractions; the amount of radioactivity changed with time (Carafoli & Crompton, 1978). This clearly indicates that a significant amount of Ca^{2+} in the mitochondria is freely exchangeable and is in equilibrium with cell Ca^{2+}. These findings together with the ability of isolated mitochondria to take up and release Ca^{2+} raised the possibility of mitochondrial buffering of cytosolic Ca^{2+}. The demonstration that isolated mitochondria can precisely maintain the extramitochondrial Ca^{2+} in the region of 0.8 μM (Nicholls, 1978b) by altering the influx or efflux components of the Ca^{2+}-translocation cycle, provides convincing evidence for the ability of mitochondria to regulate extramitochondrial Ca^{2+} *in vitro*. It also strongly supports the possibility of the organelle acting as a buffer for Ca^{2+} *in vivo*.

A large number of enzyems, both intra- and extramitochondrial, have been shown to be sensitive to very low concentrations of Ca^{2+} (Gomperts, 1976). For example, phosphorylase b kinase (Cohen, 1974), NAD-linked isocitrate dehydrogenase (Denton *et al.*, 1978) 2-oxo glutarate dehydrogenase (McCormack & Denton, 1979) and pyruvate dehydrogenase are activated (Denton *et al.*, 1975) while pyruvate kinase (Meli & Bygrave, 1972) is inhibited by the ion. Ca^{2+} is also known to enhance the oxidation of α-glycerophosphate (Hansford & Chappell, 1967) succinate (Ezawa & Ogata, 1977), β-hydroxybutyrate (Malmstrom & Carafoli, 1976) and fatty acids (Otto & Ontko, 1978). By

regulating the cytosolic Ca^{2+} concentration, mitochondria are thought to play a role in the regulation of cell metabolism. For example the *in vitro* demonstration of the mitochondria to transport Ca^{2+} and hence control protein synthesis (Rao *et al.*, 1974), phospholipid synthesis (Roberts & Bygrave, 1973) and the activity of extramitochondrial enzymes like pyruvate kinase (Meli & Bygrave, 1972) provides convincing evidence for the ability of mitochondria to regulate cell metabolism. However, the relevance of these findings to the *in vivo* situation is still unknown.

In cardiac tissue the mitochondrial transport system is thought to supplement the function of sarcoplasmic reticulum in Ca^{2+} sequestration and hence play a role in the contraction-relaxation cycle (Lehninger, 1970; Bygrave, 1977; Mela, 1977; Bygrave, 1978a,b; Carafoli & Crompton, 1978; Chapman, 1979; Fabiato & Fabiato, 1979). The role of mitochondrial Ca^{2+} transport in the beat-to-beat regulation of cytosolic Ca^{2+} is unsettled, though isolated mitochondria have been shown to be competent enough kinetically for such a role (Carafoli & Crompton, 1978). Rat heart mitochondria has been shown to remove troponin-bound Ca^{2+} *in vitro* (Carafoli, 1974).

Cells from calcifying tissues have been demonstrated to contain ultrastructural dense granules located in mitochondria containing calcium phosphate (Mela, 1977). Mitochondria isolated from these tissues have a very large

capacity to accumulate Ca^{2+} and they have been implicated in the process of calcification (Schraer et al., 1973).

AIMS OF PRESENT WORK

At the commencement of the present study knowledge on aspects of the mechanism of mitochondrial Ca^{2+} transport was still lacking. With the appearance in the literature of methods to measure the protonmotive force (Nicholls, 1974), it seemed important to attempt to more closely identify the relation of the membrane potential to Ca^{2+} movements in mitochondria. Another factor of which advantage was taken is that trialkyltin compounds like tributyltin can perturb the transmembrane pH gradient (Stockdale et al., 1970). It was felt that this probe would assist as well in providing insights into this problem. The experiments on the role of H^{+} concentration and efflux of Ca^{2+} from mitochondria evolved as the study progressed.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

Isolation of mitochondria from rat liver

Rats (Wistar albino strain) weighing approximately 200g were killed by cervical dislocation and the livers rapidly removed and placed in ice-cold isolation medium containing 250 mM-sucrose, 5 mM-Hepes, and 0.5 mM-EGTA adjusted to pH 7.4 with KOH. The livers were minced with scissors and homogenized by two passes with a glass/teflon tissue disintegrator (A.H. Thomas Co., Philadelphia, PA, U.S.A., size C)), which was motor driven at 900 rev./min. The resulting suspension was made up to 80 ml with isolation medium and mitochondria were isolated by differential centrifugation (Johnson & Lardy, 1967). The suspension was centrifuged at 900g for 5 min in a Sorvall RC-2B centrifuge fitted with a SS-34 rotor. The pellets were resuspended in the above medium and centrifuged at 800g for 5 min. The combined supernatants were centrifuged at 4500g for 5 min. The mitochondrial pellets were washed twice with EGTA-free isolation medium by resuspending and centrifuging at 4500g for 5 min. The pellet was resuspended in the EGTA-free isolation medium at a protein concentration of about 80-100 mg/ml. Mitochondria isolated by this procedure were shown to have a higher Ca^{2+} transporting ability than mitochondria prepared by standard techniques (Bygrave et al., 1978a) and were used in most of the experiments described.

Isolation of mitochondria from rat heart

Rat heart mitochondria were isolated by a slight modification of the method described by Vercesi et al. (1978). The hearts were quickly excised following cervical dislocation of the animals and thoroughly washed with ice-cold isolation medium containing 250 mM-sucrose, 5 mM-Hepes (pH 7.4 with KOH) and 0.5 mM-EGTA. They were finally minced with chilled scissors and were diluted to about 10 ml per heart with cold isolation medium containing 0.5 mg of subtilopeptidase per heart. The tissue was incubated with the enzyme for 15 min with occasional stirring in ice, after which the medium was decanted and discarded. The pellet was washed with isolation medium to remove any remaining subtilopeptidase. The finely-minced tissue was transferred to a Thomas (size C) homogenizer with about 10 ml of isolation medium per heart and was homogenized by five strokes of a motor-driven loose-fitting pestle (clearance 0.015 inch) followed by homogenization with three strokes of a tight-fitting pestle (clearance 0.007-0.009 inch). The resulting homogenate was diluted to about 20 ml per heart and centrifuged at 600g for 5 min in a Sorvall RC-2B refrigerated centrifuge fitted with a SS-34 rotor. The supernatant medium was carefully decanted and centrifuged at 10,000g for 10 min. The fluffy layer was removed and the tight pellet was washed twice by resuspending in EGTA-free isolation medium and centrifuging at 12,000g for 10 min. The final pellet was resuspended in EGTA-free isolation medium at a protein concentration of about 20-30 mg/ml.

Ca^{2+} transport

Ca^{2+} transport was measured either by using $^{45}Ca^{2+}$ or by using a Ca^{2+} ion-sensitive electrode.

$^{45}Ca^{2+}$ technique: The reaction was carried out in a water-jacketed vessel with constant stirring. The medium described in the figure legends was allowed to equilibrate to the temperature indicated in the figure legends, after which time mitochondria were added. Ca^{2+} transport was initiated by adding $^{45}CaCl_2$. The reaction was terminated at various times either (a) by quenching with EGTA and Ruthenium Red (Reed & Bygrave, 1974a; 1975a) or (b) by filtration.

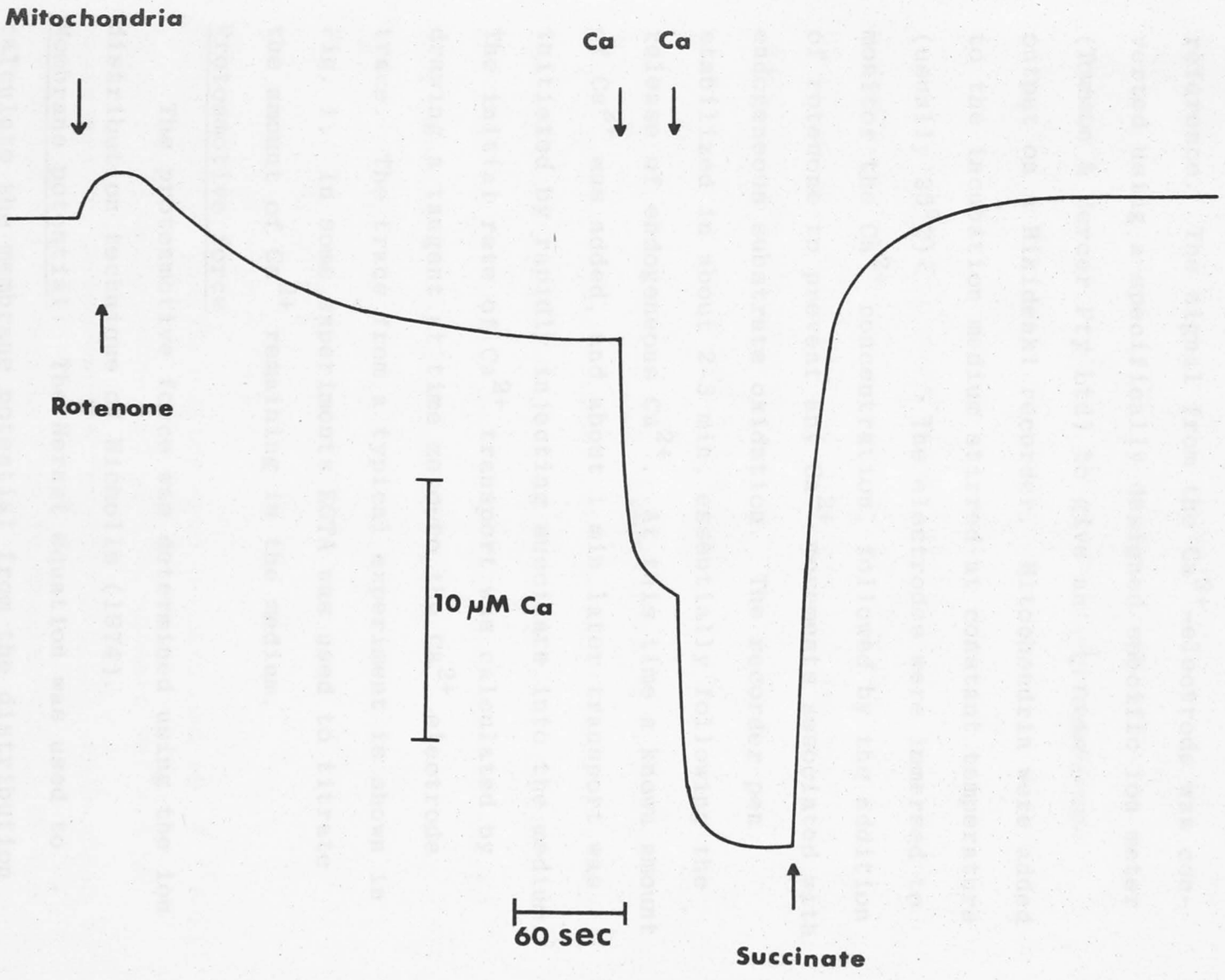
In the quench technique 100 µl aliquots of the reaction medium were transferred at appropriate time intervals into Eppendorf tubes containing ice-cold 150 mM-KCl, 3 mM-Hepes pH 7.4, 100 µM-EGTA and 2 µM-Ruthenium Red. The quenched solution was mixed thoroughly and centrifuged (Eppendorf Microfuge, 2 min at 12,000g) and 100 µl samples of the supernatant transferred to vials containing 10 ml of scintillation fluid. The amount of $^{45}Ca^{2+}$ added was determined by quenching 100 µl of the reaction medium and counting an aliquot of the quenched sample.

In the filtration technique 100 µl aliquots of the reaction medium were filtered using membrane filters and washed with 0.5 mM-EGTA containing buffered iso-osmotic sucrose solution and filter papers dissolved in 10 ml of scintillation fluid.

Ca^{2+} electrode technique: In this technique the Ca^{2+} concentration in the medium was continuously measured

Fig. 1. Ca^{2+} ion-sensitive electrode technique for monitoring mitochondrial Ca^{2+} movements

The incubation medium contained 150 mM-KCl and 3 mM-Hepes (pH 7.4). Mitochondria (1mg/ml) were added as indicated followed immediately by the addition of 1 μM-rotenone as shown. After the electrode has stabilized, 10 μM-$CaCl_2$ was added as indicated. Ca^{2+} uptake was initiated by energizing the mitochondria with 5 mM-succinate. The figure shows the linear response of the electrode to Ca^{2+}.


Mitochondria
Ca
Ca
Rotenone
10 μM Ca
60 sec
Succinate

using an ion-sensitive Ca^{2+} electrode (Radiometer, Copenhagen, F2112), with a combination pH electrode as reference. The signal from the Ca^{2+}-electrode was converted using a specifically designed specific ion meter (Townson & Mercer Pty Ltd) to give an linear output on a Rikidenki recorder. Mitochondria were added to the incubation medium stirred at constant temperature (usually 25°C). The electrodes were immersed to monitor the Ca^{2+} concentration, followed by the addition of rotenone to prevent any Ca^{2+} movements associated with endogeneous substrate oxidation. The recorder pen stabilized in about 2-3 min, essentially following the release of endogeneous Ca^{2+}. At this time a known amount of Ca^{2+} was added, and about 1 min later transport was initiated by rapidly injecting succinate into the medium. The initial rate of Ca^{2+} transport was calculated by drawing a tangent at time zero to the Ca^{2+} electrode trace. The trace from a typical experiment is shown in Fig. 1. In some experiments EGTA was used to titrate the amount of Ca^{2+} remaining in the medium.

Protonmotive force

The protonmotive force was determined using the ion distribution technique of Nicholls (1974).

Membrane potential: The Nernst equation was used to calculate the membrane potential from the distribution of $^{86}Rb^{+}$ in the presence of valinomycin.

$$\Delta E = 2.303 \frac{RT}{nF} \log \frac{[^{86}Rbi^{+}]}{[^{86}Rbo^{+}]}$$

where in ΔE = membrane potential

R = gas constant = 1.987 cal x $mole^{-1}$ x T^{-1}

T = absolute temperature

n = charge of the ion = 1

F = Faradays constant = 23,063 cal x $volt^{-1}$ x $equi^{-1}$

At 25°C $\Delta E = 59 \log \frac{[^{86}Rbi^{+}]}{[^{86}Rbo^{+}]}$

pH gradient: $[^{3}H]$ acetate distribution was used to calculate the pH gradient

$$\Delta pH = \log \frac{[A]o}{[A]i}$$

$[^{14}C]$ methyl amine was used normally as an indicator of the extra matrix volume. Under certain conditions (e.g. in the presence of uncoupler), methylamine distribution was used to calculate ΔpH, in which case acetate served as a measure of the extramatrix volume.

Δp: $\Delta p = \Delta E - Z\,\Delta pH$ where $Z = 2.303 \frac{RT}{nF}$

At 25°C, $Z = 59$

$\Delta p = \Delta E - 59\,\Delta pH$

Measurement: The incubation medium contained 10 µM $^{86}Rbcl$, 50 µM $[^{14}C]$ methylamine, 50 µM $[^{3}H]$ acetate and 0.5 µM valinomycin. Mitochondria (usually 1 mg/ml) were added to the medium and at various time intervals, 400 µl of the sample was filtered through a membrane filter (0.65 µ porosity, Millipore Corporation) using a negative pressure. The filtration was complete usually within 5 sec. The filter papers were dissolved in 10 ml of scintillation

fluid. 15 µl of the medium was spotted on to a filter paper and dissolved in 10 ml of scintillation fluid to obtain the standard counts. The maximum particle energies of the isotopes were sufficiently separate (0.018 for ^{3}H, 0.156 for ^{14}C and 1.7 MeV for $^{86}Rb^{+}$), to enable the three isotopes to be counted simultaneously with good separation on a 3-channel liquid scintillation counter.

Calculations: Corrections were made for crossover and the apparent spaces (r,c & h for $^{86}Rb^{+}$, ^{14}C & ^{3}H, respectively) occupied by each isotope on the filter paper were calculated as follows:-

$$r = 400/\frac{(400 \times {}^{86}Rb^{+}\text{ standard count})}{(15 \times {}^{86}Rb^{+}\text{ test count})} - 1$$

$$c = 400/\frac{(400 \times {}^{14}C\text{ standard count})}{(15 \times {}^{14}C\text{ test count})} - 1$$

$$h = 400/\frac{(400 \times {}^{3}H\text{ standard count})}{(15 \times {}^{3}H\text{ test count})} - 1$$

If h>c

$$\Delta E = 2.303 \frac{RT}{nF} \log \left(\frac{r-c}{m}\right)$$

$$-Z\Delta pH = 2.303 \frac{RT}{nF} \log \left(\frac{h-c}{m}\right)$$

If h<c

$$\Delta E = 2.303 \frac{RT}{nF} \log \left(\frac{r-h}{m}\right)$$

$$Z\Delta pH = 2.303 \frac{RT}{nF} \log \left(\frac{c-h}{m}\right)$$

If h=c

$$\Delta E = 2.303 \frac{RT}{nF} \log \left(\frac{r-h \text{ or } c}{m}\right)$$

$$\Delta pH = 0$$

m = volume of the mitochondrial matrix which was assumed to be 0.4 µl/mg of mitochondrial protein (Mitchell & Moyle, 1969a; Nicholls, 1974).

$$\Delta p = \Delta E - Z \Delta pH$$

Calculations were usually carried out using a UNIVAC computer programme.

Scintillation fluid

Aqueous samples were counted using a scintillation fluid containing 6gm of Butyl PBD/litre in toluene/2 - methoxy ethanol (3:2, v/v). Filter papers were dissolved in the above scintillation fluid containing 10% Biosolv BBS - 3.

Radioactive counting

$^{45}Ca^{2+}$ was counted in the ^{3}H - ^{14}C channel of a Packard Tricarb or Beckman scintillation counters LS - 100, LS - 350 or LS - 330. $^{86}Rb^{+}$, ^{14}C and ^{3}H together were counted simultaneously using the three channels of either the Packard Tricarb or the Beckman LS - 350 scintillation counters. The discriminator settings were such that crossovers of 21 and 3.6% were allowed from ^{32}P channel into the ^{14}C and ^{3}H channels respectively, and 26.8% was allowed from the ^{14}C channel into the ^{3}H channel. Background corrections were made automatically while counting.

Respiration

Mitochondrial oxygen uptake was measured polarographically using a Clark - type oxygen electrode obtained from the Yellow Springs Instrument Co. (Ohio, U.S.A.).

The medium was assumed to contain 444ng atoms of oxygen/ml.

Swelling

Mitochondria swelling was monitored semiquantitatively by continuously recording the absorbance of mitochondrial suspensions at 520 nm, using a Varion - Techtron split-beam recording spectrophotometer, with a constant temperature cell housing.

Mitochondrial phosphate transport

Mitochondrial Pi transport was measured either directly by following the accumulation of Pi by the method of Coty & Pedersen (1974) as described by Barritt et al. (1978) or indirectly by following the swelling of mitochondria in response to the accumulation of Pi in the presence of the permeant cation NH_4^+. In the former technique, mitochondria (10 mg/ml) were pre-incubated for 1 min in the medium described in the figure legends. Transport of Pi was initiated by the addition of 0.5 mM-Pi. 200 µl of the reaction mixture was removed at varying times and quenched with 1.0 ml of buffered sucrose solution containing 100 µM-PCMBS. The samples were centrifuged at 12,000g for 2 min and the supernatant removed by aspiration. The pellets were resuspended in 1.0 ml of quench medium, recentrifuged, and the supernatant removed. The pellets were disintegrated into 500 µl of 10% (w/v) trichloroacetic acid using a disintegrator and stored for 30 min on ice. The solution was centrifuged and aliquots of the supernatant were assayed

for Pi by the method of Baginski et al. (1967).

Protein determination

Mitochondrial protein was assayed by the biuret method (Layne, 1957) after solubilization with deoxycholate (Jacobs et al., 1956). Corrections were made for non-biuret colour and turbidity by subsequent cyanide treatment (Szarkowska & Klingenberg, 1963). Bovine serum albumin was used as a standard.

Radioisotopes

$^{45}CaCl_2$, $^{86}RbCl$, [^{14}C] sucrose, [^{14}C] methylamine hydrochloride and [^{3}H] acetate, were obtained from The Radiochemical Centre, Amersham, Bucks, U.K.

Chemicals

Rotenone, valinomycin, CCCP, antimycin A and oligomycin were from Sigma Chemical Co., St Louis, Missouri, U.S.A.

Tributyltin was obtained from British Drug Houses, Poole, England.

Nucleotides were from Boehringer Co., Sydney, Australia.

Standard $CaCl_2$ solution (0.1 M) was obtained from Orion Research Incorp., Cambridge, Massachusetts, U.S.A.

Butyl PBD was obtained from Koch-Light Laboratories Ltd., Bucks, England.

Biosolv - BBS - 3 was from Beckman Instruments, Fullerton, California, U.S.A.

All other chemicals used were of analytical grade.

Reproducibility of data

Each experiment was carried out at least three times. Variation between experiments did not amount to more than about 10%.

CHAPTER 3

THE INTERACTION OF TRIBUTYLTIN WITH THE MITOCHONDRIAL CALCIUM TRANSPORT SYSTEM OF RAT LIVER

THE INTERACTION OF TRIBUTYLTIN WITH THE MITOCHONDRIAL CALCIUM TRANSPORT SYSTEM OF RAT LIVER

INTRODUCTION

As already discussed in Chapter 1, it is widely accepted that compounds such as Pi and acetate enhance the capacity of the mitochondria to accumulate Ca^{2+} and stimulate the initial rate of Ca^{2+} uptake. Lehninger (1974) considers that permeant anions most effective in stimulating Ca^{2+} transport are those that possess the ability to donate a proton to the mitochondrial matrix. Those unable to stimulate Ca^{2+} uptake lack this ability. Moreover he considers that the primary event in Ca^{2+} transport is prior movement of the proton-donating anion into the matrix space. Kinetic studies concerned with the stimulation of Ca^{2+} transport by permeant anions have revealed that they do so by increasing the Vmax with little influence on the Km for Ca^{2+} transport (Reed & Bygrave, 1975b). This has led to the suggestion that anions stimulating Ca^{2+} transport enhance the dissociation of Ca^{2+} from the Ca^{2+} carrier complex on the matrix side of the inner mitochondrial membrane (Reed & Bygrave, 1975b). Further impetus to study in more detail the role of anions in Ca^{2+} transport was provided on the one hand by the recent suggestion by Moyle & Mitchell (1977a,b) that the Ca^{2+} carrier might indeed be a "calcium-phosphate symporter" in which the anion moves across the inner membrane together with the cation, and on the other, from

the report of Harris & Zaba (1977) which demonstrates an absolute requirement of a penetrant anion for Ca^{2+} transport by liver and heart mitochondria.

Further information about the role of permeant anions in mitochondrial Ca^{2+} transport might be gained by using trialkyltin compounds which mediate a Cl^-/OH^- exchange across the inner mitochondrial membrane. Trialkyltin compounds originally introduced by Aldridge (Aldridge & Cremer, 1955; Aldridge, 1958; Aldridge & Threlfall, 1961; Aldridge & Rose, 1969) are potent inhibitors of mitochondrial oxidative phosphorylation. The compounds inhibit phosphorylation coupled to respiration (Aldridge & Cremer, 1955), inhibit DNP-stimulated ATPase, but stimulate ATPase in the absence of DNP (Aldridge, 1958), inhibit respiration stimulated by arsenate but do not inhibit respiration stimulated by DNP or FCCP or by Ca^{2+}, Sr^{2+} or Mn^{2+} (Stockdale et al., 1970). The compounds stimulate respiration in the absence of ADP, but inhibit respiration in the presence of ADP (Aldridge, 1958; Sone & Hagihara, 1964; Stockdale et al., 1970) and lower mitochondrial substrate concentration (Harris et al., 1973; Skilleter, 1975). The action of trialkyltin compounds depends on the presence of appropriate anions in the incubation medium. In a Cl^--containing medium they mediate a Cl^-/OH^- exchange across the inner mitochondrial membrane (Stockdale et al., 1970; Selwyn et al., 1970b; Skilleter, 1976) which also

can be demonstrated across other natural membranes like those of the chloroplast (Watling & Selwyn, 1970), and erythrocyte (Selwyn et al., 1970b; Motais et al., 1977) as well as artificial membranes (Selwyn et al., 1970b). Trialkyltin compounds are now known to bring about three mechanistically different processes through the interaction with mitochondria (Selwyn, 1976; Aldridge et al., 1977). They are:

a) Cl^--dependent effects brought about by an exchange of intramitochondrial OH^- for extramitochondrial Cl^-,

b) Cl^--independent effects similar to those brought about by oligomycin, and

c) physical disruption of the mitochondria brought about by large-scale swelling of mitochondria.

The binding of trialkyltin compounds to mitochondria led to the assignement of two classes of binding sites, one of high affinity and the other of low affinity (Aldridge & Street, 1970). The high affinity site is thought to involve an interaction with a histidine residue (Farrow & Dawson, 1978). The structure of trialkyltin compounds are known, hence an investigation of the mode of action of these compounds unlike other inhibitors of oxidative phosphorylation, is possible.

Previously two laboratories have focused some attention on the possible effects of trialkyltin compounds on Ca^{2+} movements in mitochondria. Manger (1969) reported that triethyltin produced no change in the steady-state internal concentration of mitochondrial Ca^{2+}. Stockdale

et al. (1970) showed that tripropyltin induces massive swelling and uncoupling in the presence of Ca^{2+} and the absence of Pi, but made no direct measurements of Ca^{2+} transport. These reports thus provide little information on the effect of trialkyltin compounds on mitochondrial Ca^{2+} transport per se. In this chapter the influence of tributyltin on initial rates, steady-states and efflux of Ca^{2+} across the inner mitochondrial membrane is described.

RESULTS

Influence of tributyltin on initial rate of Ca^{2+} transport

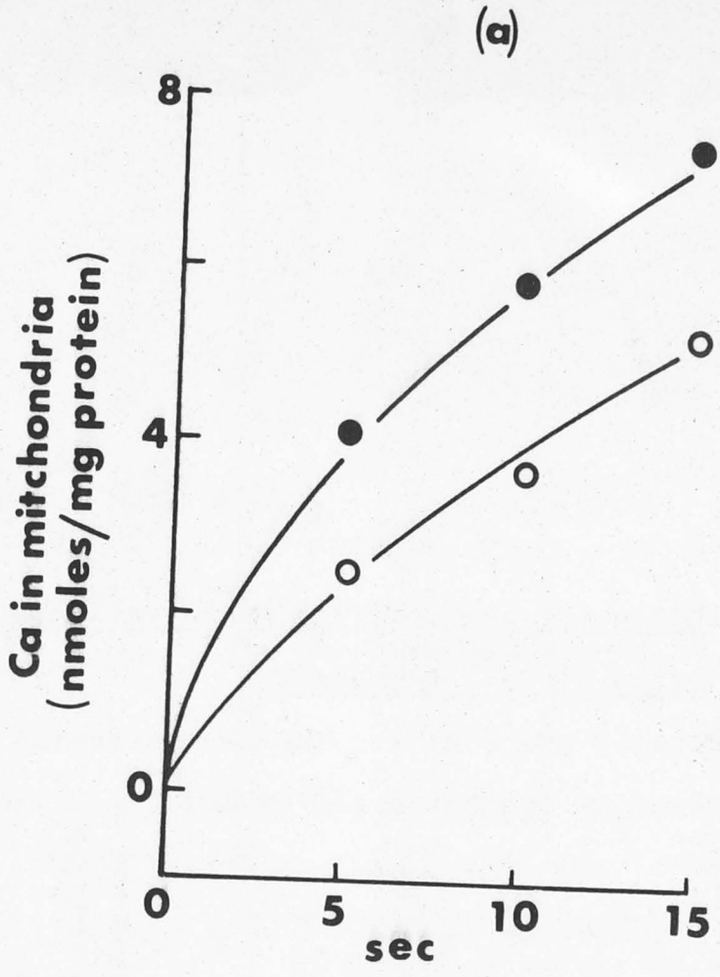
The data in Fig. 1 show the influence of a fixed low concentration of tributyltin on the initial rate of Ca^{2+} transport measured either by the EGTA-Ruthenium Red quench technique (Fig. 1a) at a low temperature($4^{o}C$) or by continuous recording of the movements of Ca^{2+} using a Ca^{2+} ion-specific electrode (Fig. 1b) at $25^{o}C$. In each experiment the initial rate of Ca^{2+} transport is increased substantially in the presence of tributyltin.

The effect of increasing concentrations of tributyltin on the initial rate of Ca^{2+} transport as measured by the quench technique is shown in Fig. 2a. As the concentration of the compound is raised to about 4 μM an increase occurs in the initial rate of Ca^{2+} transport; maximal stimulation is approx. 130% at 4μ M-tributyltin. Increasing the concentration of tributyltin further leads to a fall-off in the stimulation. The data in Fig. 2a also show that tributyltin has no effect on the initial rate of Ca^{2+} transport in a Cl^{-}-free medium (KNO_3 medium).

When Ca^{2+} transport was measured by the Ca^{2+}-electrode technique (Fig. 2b) qualitatively similar results were obtained. Maximal stimulation occurred at about 0.5 μM-tributyltin in the experiment shown and higher concentrations produced less stimulation as seen in Fig. 2a. In some experiments with this technique, maximal stimulation

Fig. 1. Effect of tributyltin on initial rate of Ca^{2+} transport by rat liver mitochondria

The reaction mixture in (a), contained 150 mM-KCl, 40 mM-Hepes (pH 7.4) 2.5 mM-succinate, and 1 mg of mitochondrial protein in a final volume of 400 µl. The reaction was started by adding 10 nmol of $^{45}Ca^{2+}$ and allowed to proceed at 4°C for the time indicated. For details see Experimental Section. Control (○), 2 µM-tributyltin (●). In the experiment in (b), the reaction mixture contained 150 mM-KCl, 3 mM-Hepes (pH 7.4), 5 mM-succinate, 1 µM-rotenone, 12.5 µM-Ca^{2+} and 2 mg of mitochondrial protein in a final volume of 4 ml at 25°C. Control (○), 0.5 µM-tributyltin (●).


(a)
8
4
0
Ca in mitchondria
(nmoles/mg protein)
0
5
10
15
sec

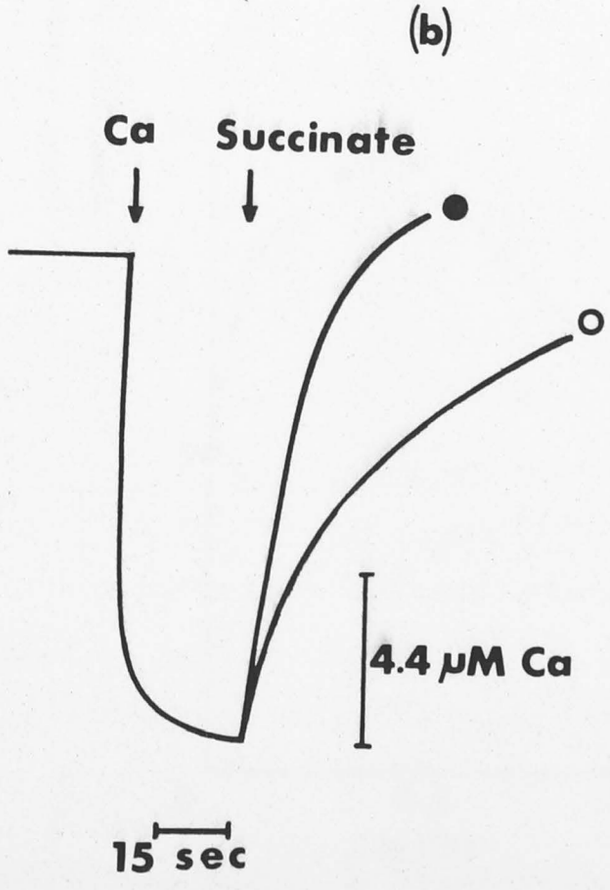
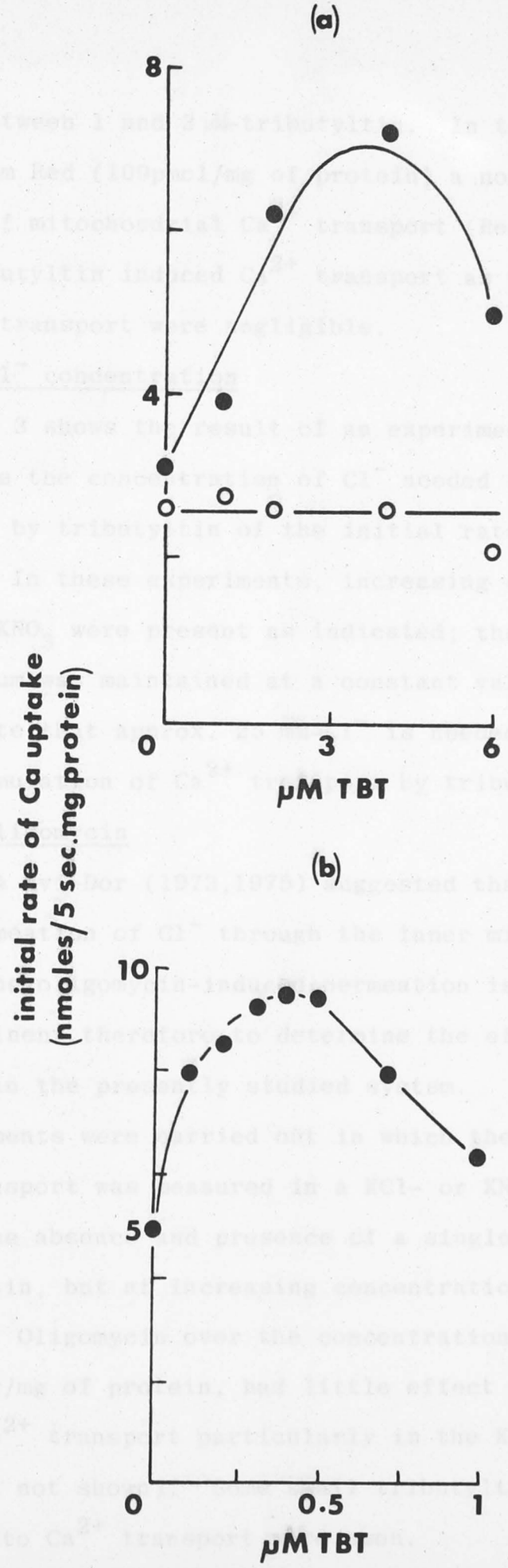

(b)
Ca
Succinate
4.4 μM Ca
15 sec

Fig. 2. Effect of tributyltin concentration on initial rates of Ca^{2+} transport

For details see Fig. 1. The concentration of tributyltin was varied as indicated. 150 mM-KCl (●), 150 mM-KNO_3 (○).

(a) $^{45}Ca^{2+}$ technique

(b) Ca^{2+} electrode technique


(a)
8
4
0
3
6
μM TBT
initial rate of Ca uptake
(nmoles/5 sec.mg protein)
(b)
10
5
0
0.5
1
μM TBT

occurred between 1 and 3 μM-tributyltin. In the presence of Ruthenium Red (100pmol/mg of protein) a non-competitive inhibitor of mitochondrial Ca^{2+} transport (Reed & Bygrave, 1974a) tributyltin induced Ca^{2+} transport as well as the basal Ca^{2+} transport were negligible.

Effect of Cl^- concentration

Figure 3 shows the result of an experiment designed to determine the concentration of Cl^- needed for maximal stimulation by tributyltin of the initial rate of Ca^{2+} transport. In these experiments, increasing concentrations of KCl and KNO_3 were present as indicated; the osmolarity of the medium was maintained at a constant value. The data indicate that approx. 25 mM-Cl^- is needed to obtain maximal stimulation of Ca^{2+} transport by tributyltin.

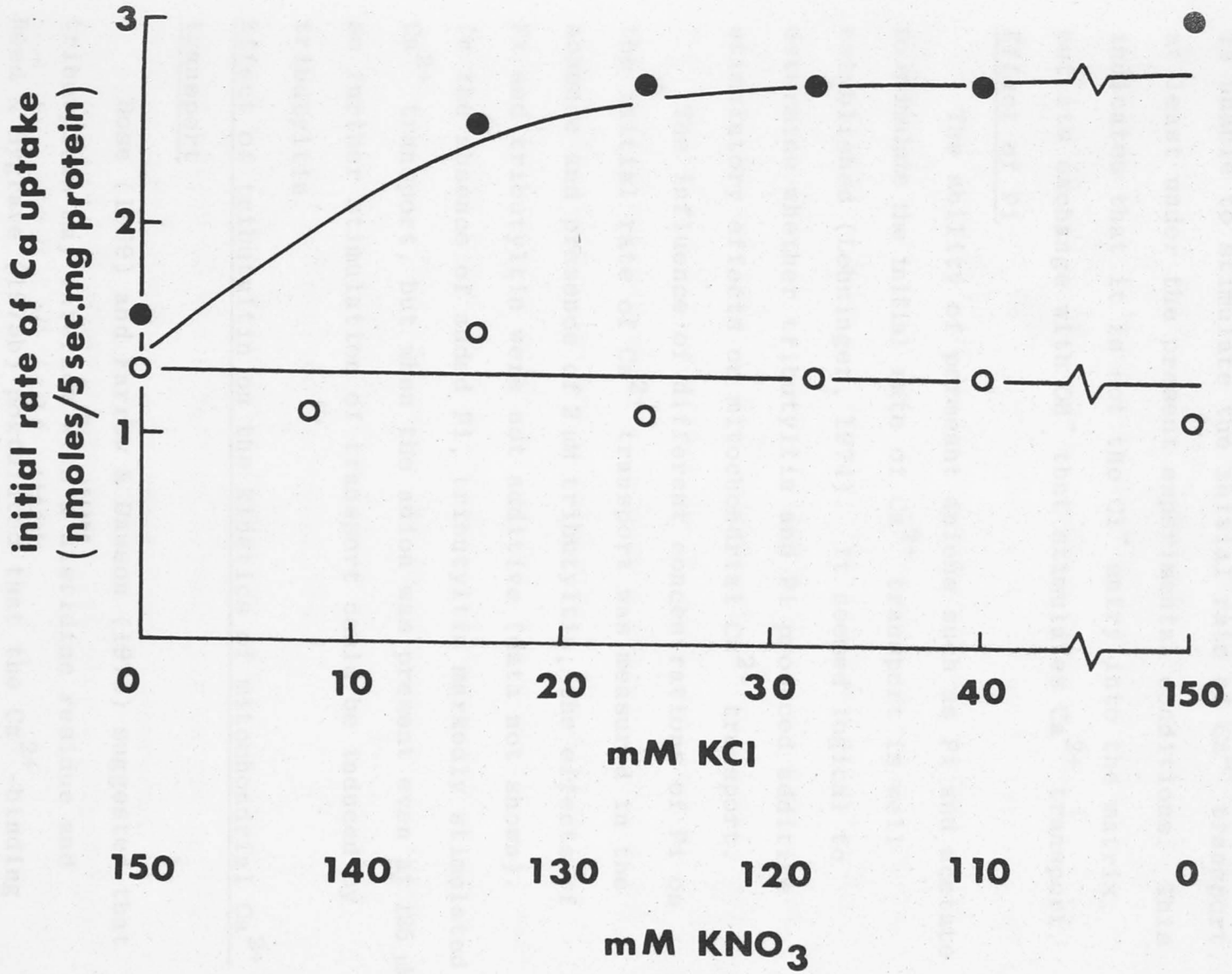
Effect of oligomycin

Ariel & Avi-Dor (1973,1975) suggested that oligomycin induces permeation of Cl^- through the inner mitochondrial membrane; the oligomycin-induced permeation is uniport. It was pertinent therefore to determine the effect of oligomycin in the presently studied system.

Experiments were carried out in which the initial rate of Ca^{2+} transport was measured in a KCl- or KNO_3-containing medium in the absence and presence of a single concentration of tributyltin, but at increasing concentrations of oligomycin. Oligomycin over the concentration range 0.25 - 10 μg/mg of protein, had little effect on mitochondrial Ca^{2+} transport particularly in the KCl-containing medium (data not shown). Some small tributyltin-independent changes to Ca^{2+} transport were seen.

Fig. 3. Effect of Cl^- concentration on the initial rate of Ca^{2+} transport measured in the absence and presence of tributyltin

For details see Fig. 1a. The concentration of KCl and KNO_3 were varied as indicated. Control (○), 4 μM-tributyltin (●).


initial rate of Ca uptake
(nmoles/5sec.mg protein)
3
2
1
0
10
20
30
40
150
mM KCl
150
140
130
120
110
0
mM KNO3

These findings suggest that any Cl^- permeation that may be induced by oligomycin (Ariel & Avi-Dor, 1973,1975) is unable to stimulate the initial rate of Ca^{2+} transport, at least under the present experimental conditions. This indicates that it is not the Cl^- entry into the matrix, but its exchange with OH^- that stimulates Ca^{2+} transport.

Effect of Pi

The ability of permeant anions such as Pi and acetate to stimulate the initial rate of Ca^{2+} transport is well established (Lehninger, 1974). It seemed logical to determine whether tributyltin and Pi produced additive stimulatory effects on mitochondrial Ca^{2+} transport.

The influence of different concentrations of Pi on the initial rate of Ca^{2+} transport was measured in the absence and presence of 2 μM tributyltin; the effects of Pi and tributyltin were not additive (data not shown). In the absence of added Pi, tributyltin markedly stimulated Ca^{2+} transport, but when the anion was present even at 125 μM no further stimulation of transport could be induced by tributyltin.

Effect of tributyltin on the kinetics of mitochondrial Ca^{2+} transport

Rose (1969) and Farrow & Dawson (1978) suggested that tributyltin may interact with a histidine residue and Reed & Bygrave (1975b) postulated that the Ca^{2+}-binding sites on the Ca^{2+} carrier also may involve a histidine residue. Because some of the effects reported above may be due to a direct interaction of tributyltin with the

Ca^{2+} carrier, the influence of tributyltin on the kinetics of mitochondrial Ca^{2+} transport was examined.

Data in Fig. 4a show the effect of 500 nM-tributyltin on the initial rate of Ca^{2+} transport measured in KCl medium at $5^{o}C$ in the presence of 0.5 - 10 μM-free Ca^{2+} maintained using NTA buffer (Reed & Bygrave, 1975a). The initial rate of Ca^{2+} transport is a sigmoidal function of the external free Ca^{2+} both in the presence and absence of tributyltin. The Km for Ca^{2+} transport is about 3 μM and was not influenced by tributyltin; the Vmax is enhanced two-fold (Fig. 4b). This indicates that the enhancement of the initial rate of Ca^{2+} transport by tributyltin was not due to any direct effect on the Ca^{2+} carrier, a conclusion reached earlier by Reed & Bygrave (1975b) for the stimulation of the initial rate by Pi. The Hill plot (Fig. 4c) provides evidence for positive cooperativity with a Hill coefficient of 1.8 which was little affected by tributyltin. This indicates the involvement of at least two interacting sites in the transport of Ca^{2+} (Bygrave et al., 1971; Vinogradov & Scarpa, 1973).

All of the information presented above involved determination of the effect of tributyltin on the initial rate of Ca^{2+} transport. It was important therefore to determine if the stimulation persisted over a longer time period.

Fig. 4. Effect of tributyltin on the kinetics of mitochondrial Ca^{2+} transport

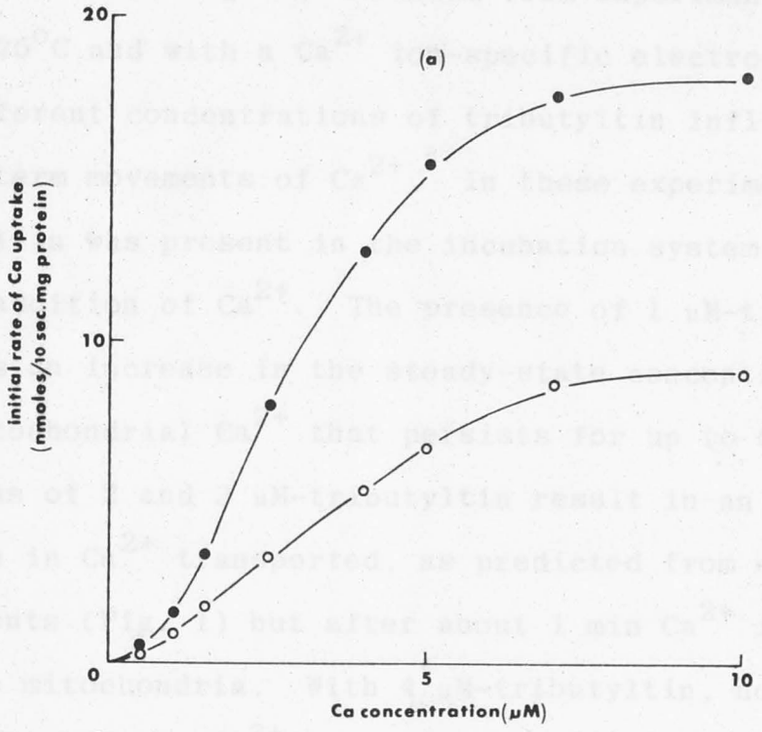
The incubation medium was the same as in Fig. 1a except that 10 mM-NTA was present. The temperature was $5^{o}C$ and the free Ca^{2+} concentration was varied from 0-10 μM. Mitochondrial concentration was 1mg/ml. Ca^{2+} transport was followed as described in the Experimental Section using $^{45}Ca^{2+}$.

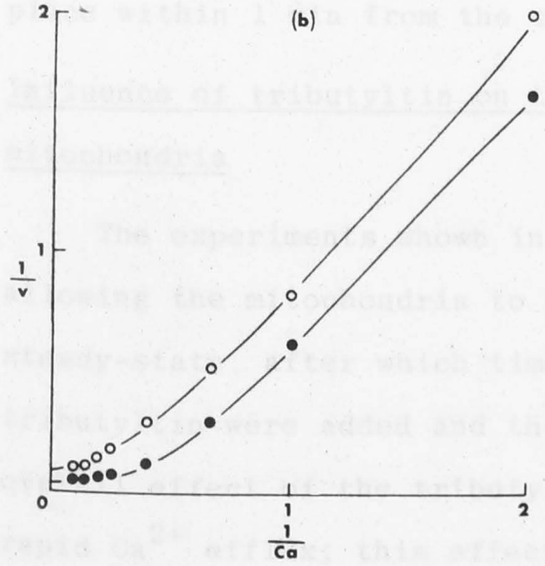
(a) Initial rate of Ca^{2+} transport as a function of free Ca^{2+} concentration in the presence (●) or absence (O) of 0.5 μM-tributyltin.

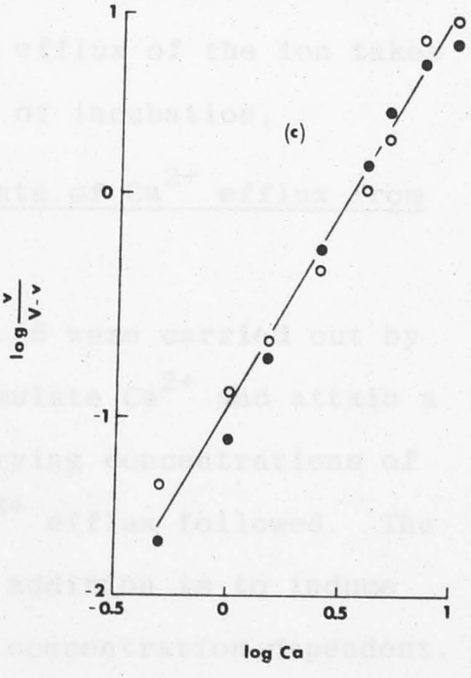
(b) Double-reciprocal plots of (a) v = nmoles Ca^{2+} transported/10 sec. mg protein. Ca = μM free Ca^{2+}.

(c) Hill plots of the data of (a).

μM.


(a)
20
10
0
5
10
initial rate of Ca uptake
(nmoles/10 sec.mg protein)
Ca concentration(μM)


(b)
2
1
0
1
2
1/v
1/Ca


(c)
1
0
-1
-2
-0.5
0
0.5
1
log v/V-v
log Ca

Influence of tributyltin on the steady-state movement of Ca^{2+}

The data in Fig. 5, obtained from experiments carried out at 25°C and with a Ca^{2+} ion-specific electrode show how different concentrations of tributyltin influence the longer-term movements of Ca^{2+}. In these experiments, tributyltin was present in the incubation system prior to the addition of Ca^{2+}. The presence of 1 μM-tributyltin produces an increase in the steady-state concentration of intramitochondrial Ca^{2+} that persists for up to 8 min. Additions of 2 and 3 μM-tributyltin result in an initial increase in Ca^{2+} transported, as predicted from earlier experiments (Fig. 1) but after about 1 min Ca^{2+} is lost from the mitochondria. With 4 μM-tributyltin, no change in initial rate of Ca^{2+} transport is observed, the mitochondria transport less Ca^{2+}, and efflux of the ion takes place within 1 min from the onset of incubation.

Influence of tributyltin on the rate of Ca^{2+} efflux from mitochondria

The experiments shown in Fig. 6 were carried out by allowing the mitochondria to accumulate Ca^{2+} and attain a steady-state, after which time varying concentrations of tributyltin were added and the Ca^{2+} efflux followed. The overall effect of the tributyltin addition is to induce rapid Ca^{2+} efflux; this effect is concentration-dependent. With low concentrations of tributyltin, Ca^{2+} efflux does

Fig. 5. Effect of tributyltin concentration on the steady-state concentration of transported Ca^{2+}

For details see Fig. 1b. The concentrations of tributyltin present before addition of succinate were 0 (○), 1 (●), 2 (△), 3 (▲) and 4 (■) μM.

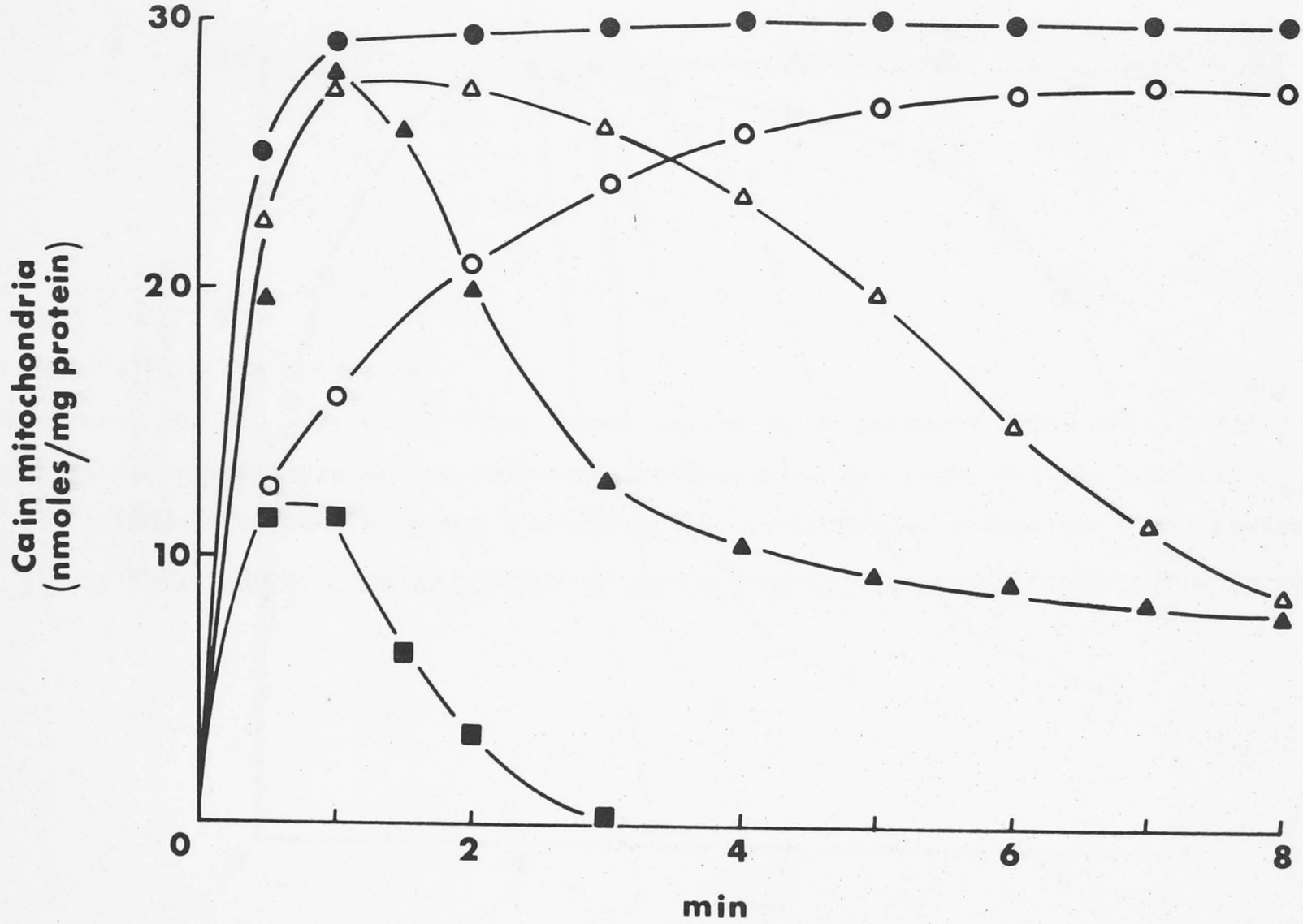
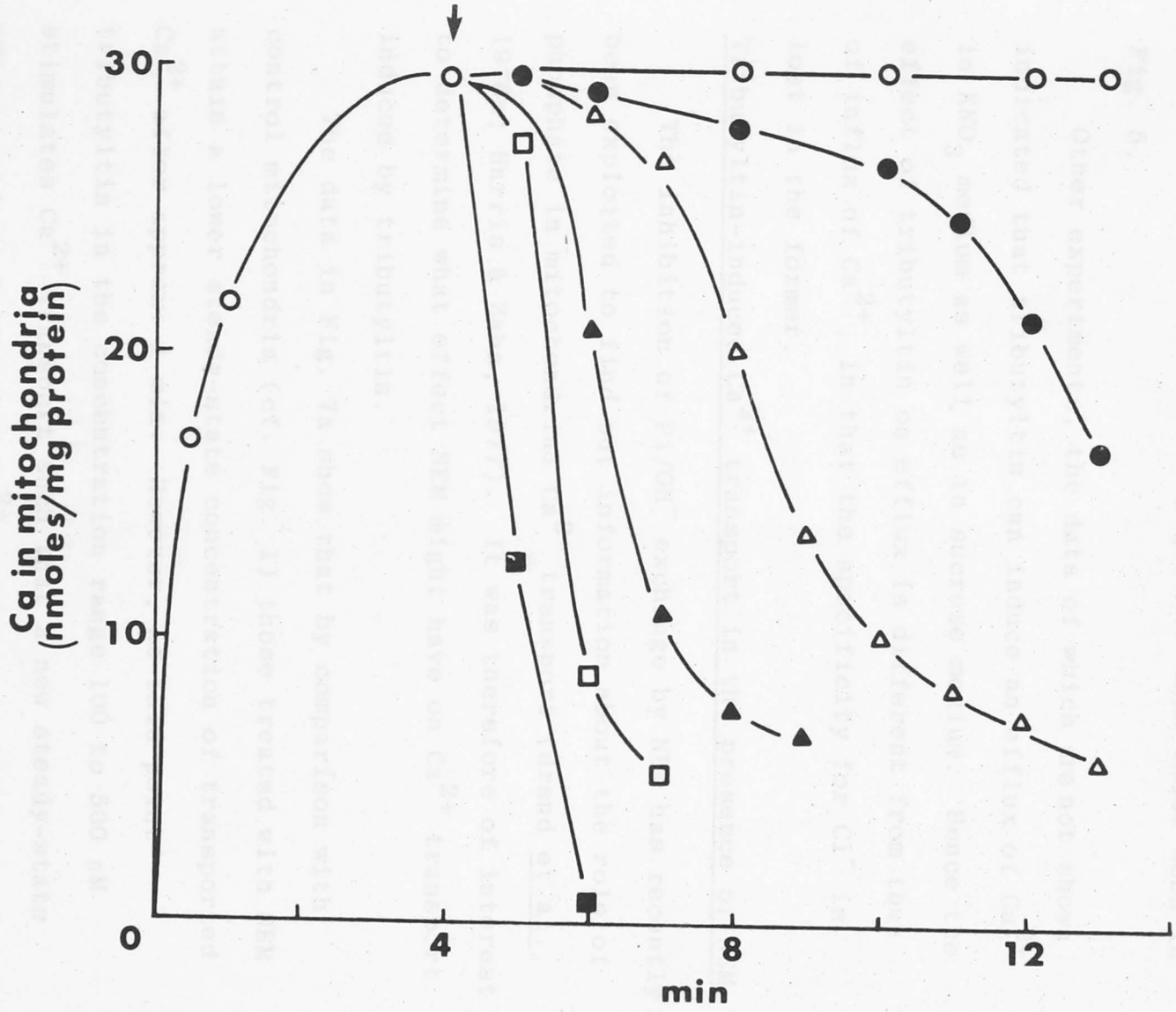

Ca in mitochondria
(nmoles/mg protein)
30
20
10
0
2
4
6
8
min

Fig. 6. Effect of tributyltin concentration on the rate of efflux of Ca^{2+} from rat liver mitochondria

Ca^{2+} (12.5 μM) was allowed to reach a steady-state concentration inside the mitochondria and after 4 min (arrow) tributyltin at the concentration indicated was added and the rate of Ca^{2+} efflux was followed using the Ca^{2+}-electrode. The concentration of tributyltin added was 0 (○), 1 (●), 2 (△), 3 (▲), 4 (□) and 6 (■) μM.


30
20
10
0
4
8
12
min
Ca in mitochondria
(nmoles/mg protein)

not occur immediately but only after several minutes have elapsed. With 3 to 6 μM-tributyltin, maximal rates of Ca^{2+} efflux occur within about 1 min. These rates of efflux also are considerably greater than those seen in Fig. 5.

Other experiments, the data of which are not shown indicated that tributyltin can induce an efflux of Ca^{2+} in KNO_3 medium as well as in sucrose medium. Hence the effect of tributyltin on efflux is different from that of influx of Ca^{2+}, in that the specificity for Cl^- is lost in the former.

Tributyltin-induced Ca^{2+} transport in the presence of NEM

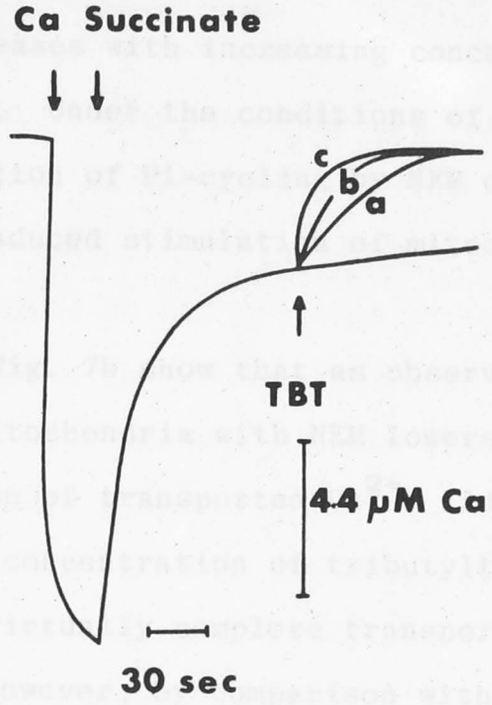
The inhibition of Pi/OH^- exchange by NEM has recently been exploited to find out information about the role of phosphate in mitochondrial Ca^{2+} transport (Brand et al., 1976a; Harris & Zaba, 1977). It was therefore of interest to determine what effect NEM might have on Ca^{2+} transport induced by tributyltin.

The data in Fig. 7a show that by comparison with control mitochondria (cf. Fig. 1) those treated with NEM attain a lower steady-state concentration of transported Ca^{2+} after approx. 1 min. However, at this point tributyltin in the concentration range 100 to 500 nM stimulates Ca^{2+} transport such that a new steady-state concentration of internal Ca^{2+} is reached. This steady-state concentration, which can be maintained for prolonged periods, is common to all additions of tributyltin in this experiment despite the fact that the initial rate

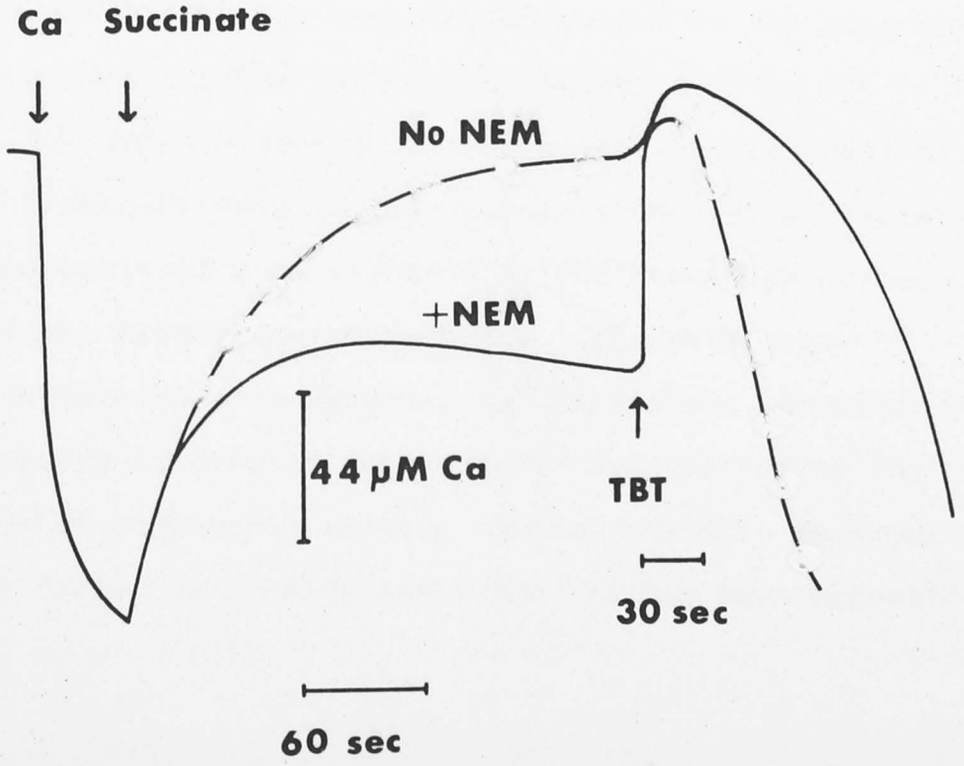
Fig. 7. Effect of tributyltin on Ca^{2+} movements in NEM-treated mitochondria from rat liver

Experimental details were as described for Fig. 1b except that NEM was present at 40 nmol/mg of protein as indicated. (a) NEM was added to the mitochondria contained in the reaction medium 2 min prior to the addition of the Ca^{2+}. Tributyltin was added (arrow) at the following concentrations: curve a, 100 nM; curve b, 200 nM; curve c, 500 nM (b) NEM was added as in Fig. 7a and after approx. 4 min 2 μM-tributyltin was added (arrow).

(a)


Ca Succinate
c
b
a
TBT
4.4 μM Ca
30 sec

(b)


Ca Succinate
No NEM
+NEM
4.4 μM Ca
TBT
30 sec
60 sec

of transport increases with increasing concentrations of added tributyltin. Under the conditions of this experiment, therefore, prevention of Pi-cycling by NEM does not impede the tributyltin-induced stimulation of mitochondrial Ca^{2+} transport.

The data in Fig. 7b show that as observed previously, pretreatment of mitochondria with NEM lowers the steady-state concentration of transported Ca^{2+}. Addition of a relatively high concentration of tributyltin, viz. 2 μM, rapidly leads to virtually complete transport of all of the added Ca^{2+}. However, by comparison with the control mitochondria, the rate of Ca^{2+} efflux is slower by about 70% (cf. Fig. 6).

DISCUSSION

The present study provides evidence that low concentration of tributyltin stimulate the initial rate of Ca^{2+} transport by mitochondria isolated from rat liver. The stimulation attributed to the presence of tributyltin was shown to depend on the concentration of Cl^- in the incubation medium, with maximal effects when the Cl^- concentration exceeded approx. 25 mM. The degree of stimulation was also related to the concentration of tributyltin added, with maximal effect at a concentration of approx. 4 μM. This concentration is very similar to that required to inhibit many of the energy-transducing reactions (Stockdale et al., 1970; Aldridge et al., 1977) and to that required for inhibition of pyruvate transport (Skilleter, 1975) and adenine nucleotide transport (Harris et al., 1973). Also this concentration causes maximal Cl^-/OH^- exchange as measured with a pH electrode (data not shown) and associated changes in the components of the protonmotive force (see Chapter 5, Fig. 3).

The effects of tributyltin on the initial rate of Ca^{2+} transport seen in the present study can be explained on the basis of a facilitated Cl^-/OH^- exchange across the inner mitochondrial membrane. The action of tributyltin on mitochondrial Ca^{2+} transport supported by the energy of respiration is quite different from any action of oligomycin in this system. This is an important point since, as already mentioned, it has been suggested

that under certain conditions trialkyltin compounds have an oligomycin-like action on mitochondrial energy transduction (Stockdale et al., 1970; Selwyn et al., 1970b; Aldridge et al., 1977).

The influence of tributyltin on the release of Ca^{2+} from mitochondria differs in two major respects from its effects on the initial rate of influx. First, higher concentrations of the compound are required with no indications of an inhibitory effect at concentrations greater than maximal. Second, tributyltin will induce a release of transported Ca^{2+} from mitochondria both in Cl^- and ion-free medium. Because of the loss of specificity for Cl^-, the release process probably involves a general disruption to the inner membrane permeability as a result of the uncoupling action of tributyltin at these concentrations (Stockdale et al., 1970; Aldridge et al., 1977); indeed swelling experiments carried out under identical conditions revealed that swelling of mitochondria accompanies efflux of Ca^{2+} (data not shown).

Further evidence that tributyltin-induced influx and efflux are mechanistically different is provided by the experiments with NEM-treated mitochondria (Fig. 7). Here it was seen that prevention of Pi-cycling by NEM did not impede the effects of tributyltin in stimulating the initial rate of Ca^{2+} transport but did considerably impede the rate at which Ca^{2+} was released from the mitochondria. The data suggest that besides being necessary for phosphoenolpyruvate-induced Ca^{2+} release

(Peng et al., 1974) Pi-cycling also enhances tributyltin-induced Ca^{2+} release from mitochondria.

The action of tributyltin described herein thus may be compared with that of Pi on mitochondrial Ca^{2+} transport. As already pointed out, this anion stimulates both the initial rate and extent of Ca^{2+} transport; such stimulation is dependent on the movement of the anion into the matrix space on the Pi transporter and involves a Pi/OH^- exchange (Coty & Pedersen, 1974).

Ca^{2+} enters the matrix of the mitochondria for different reasons but a balancing pair of negative charges or chelation will always be required. The negative charges may be provided by:-

a) the limited amount of OH^- in the medium matrix;

b) various fixed anionic sites in the alkaline environment of the matrix;

c) the presence of permeant anions (Pi, arsenate, acetate, β-hydroxybutyrate, lactate and CO_2; Lehninger, 1974); Pi has the added effect of forming an insoluble compound with Ca^{2+}, so both are removed and the gradient can be maintained; and

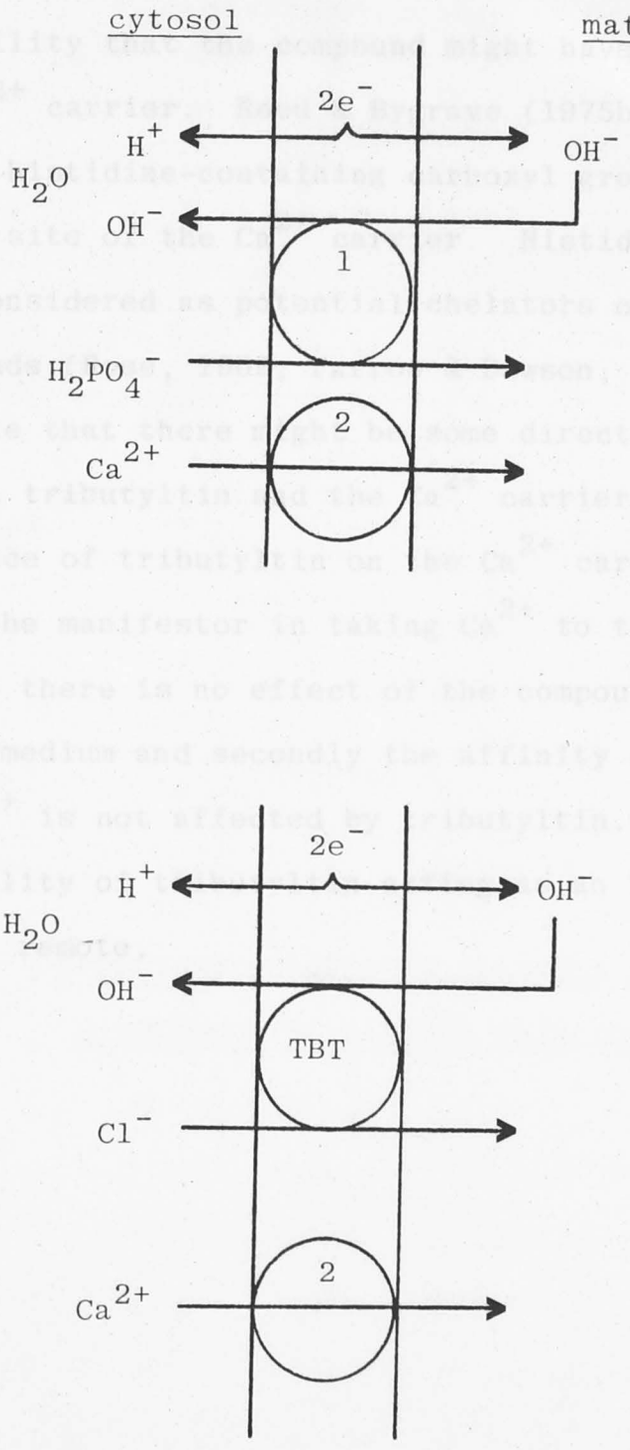
d) chloride in the presence of trialkyltin compounds.

The primary act is the charge separation resulting in OH^- on the matrix side of the inner mitochondrial membrane and extrusion of H^+ to the outside. The rate of entry of Ca^{2+} will therefore equal the rate of OH^- generation less the rate of OH^- leakage (or H^+ entry) plus the rate of generation of any unknown negative sites or Ca^{2+}-

chelating sites in the matrix. The negative charges or the chelating sites do not necessarily require synthesis of new molecules but may be due to conformational changes, perhaps stimulated by the alkaline environment in the matrix. The cumulative effects of the different sites attracting Ca^{2+} in the absence of added anions are responsible for a limited Ca^{2+} content (Fig. 2).

The effects of Pi and Cl^- in the presence of trialkyltin are of particular interest when considered in relation to the classes of anions capable of stimulating Ca^{2+} transport. As mentioned in the introduction, Lehninger (1974) has shown that the permeant anions (Pi, acetate, etc.) most effective in stimulating Ca^{2+} transport are those that have the potential ability to donate a H^+ to the mitochondrial matrix. The permeant anions which do not stimulate Ca^{2+} uptake lack the ability to donate a H^+ to the matrix and evidently are unable to exchange across the mitochondrial membrane to enter the matrix by exchange for a OH^- (NO_3^-, CNS^-, ClO_3^-, SCN^- and Cl^-, in the absence of trialkyltin). The trialkyltin-facilitated Cl^-/OH^- exchange is clearly as effective as the permeant anion Pi in stimulating the initial rate of Ca^{2+} transport. Since trialkyltins are quite specific for halides, these compounds do not result in the entry of increased Ca^{2+} in the presence of NO_3^-. These releationships are schematically represented in Scheme 1.

Though the effects of tributyltin can be satisfactorily explained by the Cl^-/OH^- exchange, there remains the



1 - Pi carrier

2 - Ca^{2+}-carrier

TBT - tributyltin

Scheme 1

Schematic representation of the effect of Pi and tributyltin

possibility that the compound might have some effect on the Ca^{2+} carrier. Reed & Bygrave (1975b) have postulated that a histidine-containing carboxyl group may be at the active site of the Ca^{2+} carrier. Histidine groups have been considered as potential chelators of trialkyltin compounds (Rose, 1969; Farrow & Dawson, 1978). So it is possible that there might be some direct interaction between tributyltin and the Ca^{2+} carrier. However, any influence of tributyltin on the Ca^{2+} carrier seems unlikely to be the manifestor in taking Ca^{2+} to the matrix since, firstly there is no effect of the compound in NO_3^- instead of Cl^- medium and secondly the affinity of the carrier for Ca^{2+} is not affected by tributyltin. Also the possibility of tributyltin acting as an ionophore for Ca^{2+} is remote.

CHAPTER 4

EFFECT OF SULPHYDRYL REAGENTS ON MITOCHONDRIAL CALCIUM TRANSPORT

EFFECT OF SULPHYDRYL REAGENTS ON MITOCHONDRIAL CALCIUM TRANSPORT

INTRODUCTION

Respiring mitochondria eject H^+. The H^+:O ratio and the H^+:site ratio traditionally have been calculated by the oxygen-pulse method carried out first by Mitchell & Moyle (1967). The technique led to a value of about 2.0 for the H^+:site ratio. This value has since been confirmed in many laboratories (see Lehninger et al., 1978a for a review). It was noted that with the oxygen-pulse method higher values can be obtained at a lower temperature presumably due to decreased endogeneous Pi movements (Brand et al., 1976b).

Rossi & Lehninger (1964) demonstrated that during stimulation of respiration by Ca^{2+}, about 2.0 Ca^{2+} ions are accumulated for each pair of electrons traversing each of the three energy-conserving sites. Saris (1963) first showed that during respiratory stimulation by Ca^{2+}, H^+ are ejected into the medium. In the absence of any anions influencing Ca^{2+} transport, about one H^+ was ejected for the uptake of one Ca^{2+} ion (Drahota et al., 1965; Rossi et al., 1966a). The H^+:Ca^{2+} ratio has been shown to vary with the Na^+, K^+ and H^+ concentration of the incubation medium (Lehninger et al., 1967). The H^+:Ca^{2+} ratio is also decreased by anions like Pi and acetate (Chance, 1965; Rasmussen et al., 1965; Rossi et al., 1966a). Since normally about one H^+ is ejected for the transport of one Ca^{2+} ion there appears to be an incomplete charge

compensation. From the Ca^{2+}: site ratio and H^+:Ca^{2+} ratio, the H^+:site ratio of 2.0 was calculated, in agreement with the values reported by Mitchell & Moyle (1967) by the oxygen-pulse method. By contrast, other workers (Cockrell et al., 1966; Massari & Azzone, 1970) observed that the number of K^+ ions transported per site (K^+:site ratio) in respiring mitochondria, significantly exceeded 2.0. Moreover Rottenberg (1970) showed that the H^+:site ratio of 2.0 is too low to provide sufficient energy for ATP synthesis. This has led to a re-examination of the H^+:site ratio.

Brand et al. (1976a) showed that in the presence of monocarboxylic acids, about four acid molecules accompanied two Ca^{2+} ions for each site indicating a H^+:site ratio of 4.0. Brand et al. (1976b) also showed that with oxygen-pulse experiments, movements of endogeneous Pi were responsible for the underestimation of the H^+:site ratio. They used NEM to inhibit Pi movements and obtained a value for the H^+:site ratio of about 3.0.

Case (1975) showed that there is no direct interaction between Pi and the divalent cation carrier. Reed & Bygrave (1975b) showed that Pi does not affect the affinity of the Ca^{2+} carrier for the ion. Hutson (1977) used NEM to inhibit Pi movements and showed that NEM does not affect the Km for the transport of Ca^{2+}, but suggested that the compound acts close to the Ca^{2+} carrier. Moyle & Mitchell (1977a,b) suggested the existence of a NEM-insensitive 'calcium-phosphate symporter'. Harris & Zaba (1977) showed

that rat liver and rat heart mitochondria transport little Ca^{2+} in the presence of NEM and oligomycin. Ca^{2+} transport by inside out submitochondrial particles appears to be absolutely dependent on Pi (Wehrle & Pedersen, 1979).

Pi enters the mitochondria via two carriers (LaNoue & Schoolwerth, 1979), an NEM-sensitive Pi/OH^- exchange accounting for more than 90% of transport and an n-butyl malonate-sensitive Pi/dicarboxylate exchange (Johnson & Chappell, 1973; Coty & Pedersen, 1974,1975). The Km for both the carriers appears to be the same. The transport of Pi through the NEM-insensitive calcium-phosphate symporter and an alternative NEM-insensitive but atractyloside-sensitive adenine nucleotide-phosphate carrier (Reynafarje & Lehninger, 1978a) remains to be established.

Hence the effect of -SH reagents that influence Pi transport was studied with the intention to gain more information about Ca^{2+} movements across the inner mitochondrial membrane. We studied specifically the $H^+:Ca^{2+}$ stoichiometry as well as the long-term movements of Ca^{2+} as influenced by the thiol-specific reagents.

RESULTS

H^+:Ca^{2+} stoichiometry

Data in Fig. 1 show a continuous recording of the Ca^{2+} and H^+ concentration in the medium with a Ca^{2+} and pH electrode. In these experiments mitochondria were deenergized by incubation with rotenone until no more Ca^{2+} and H^+ movements occur, followed by the addition of Ca^{2+} as indicated. Ca^{2+} transport was initiated by rapidly injecting sodium succinate the pH of which was adjusted to that of the medium. The responses of the Ca^{2+} and H^+ electrodes were recorded continuously. Entry of Ca^{2+} (decrease in medium concentration) into mitochondria is associated with ejection of H^+ and the stoichiometry between the amount of H^+ ejected with Ca^{2+} accumulated was about 1.0-1.2. This finding essentially confirms earlier observations by other workers (see Lehninger et al., 1967 for a review).

Effect of NEM on H^+:Ca^{2+} stoichiometry

The above experiment was repeated in the presence of NEM (40 nmol/mg of protein) to inhibit movements of Pi and the results are shown in Fig. 2. In this experiment we observed that during energization of mitochondria with succinate there was considerable H^+ ejection which was not inhibited by Ruthenium Red and hence is unrelated to Ca^{2+} movements. This is also shown by the Ca^{2+}-electrode recording which indicates that at the concentration of Ruthenium Red employed there was little decrease in medium Ca^{2+}. Similar Ruthenium Red-insensitive H^+

Fig. 1. H^+: Ca^{2+} stoichiometry

Mitochondria (1mg/ml) were equilibrated at 25°C in a medium containing 150 mM-KCl, 3 mM-Hepes (pH 7.4 with KOH) and 1 µM-rotenone. 25 µM-$CaCl_2$ was added as shown in the figure followed by the addition of 5 mM-succinate as indicated. Ca^{2+} and H^+ movements were followed using a Ca^{2+} and pH electrode as described in the Experimental Section.

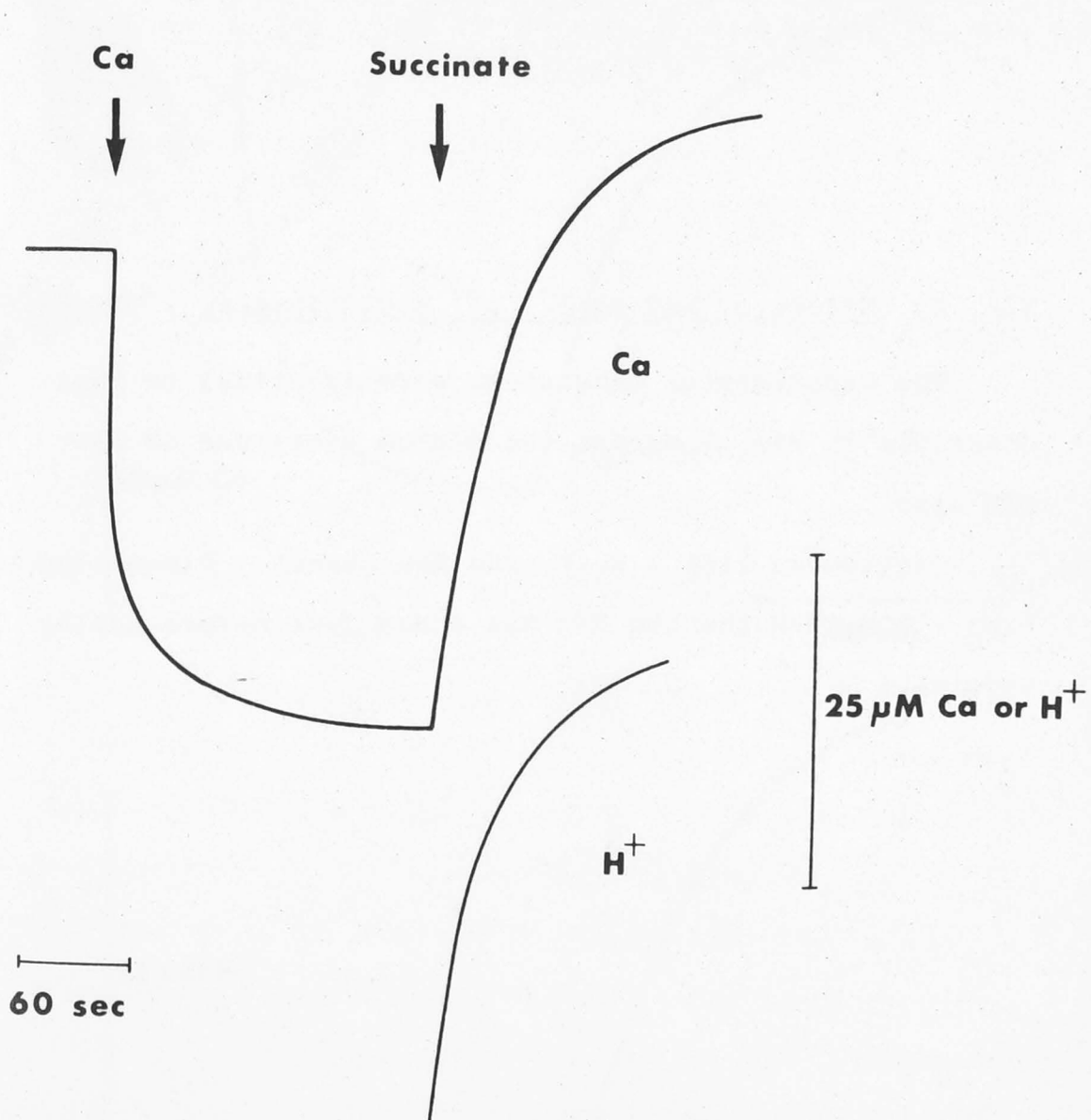
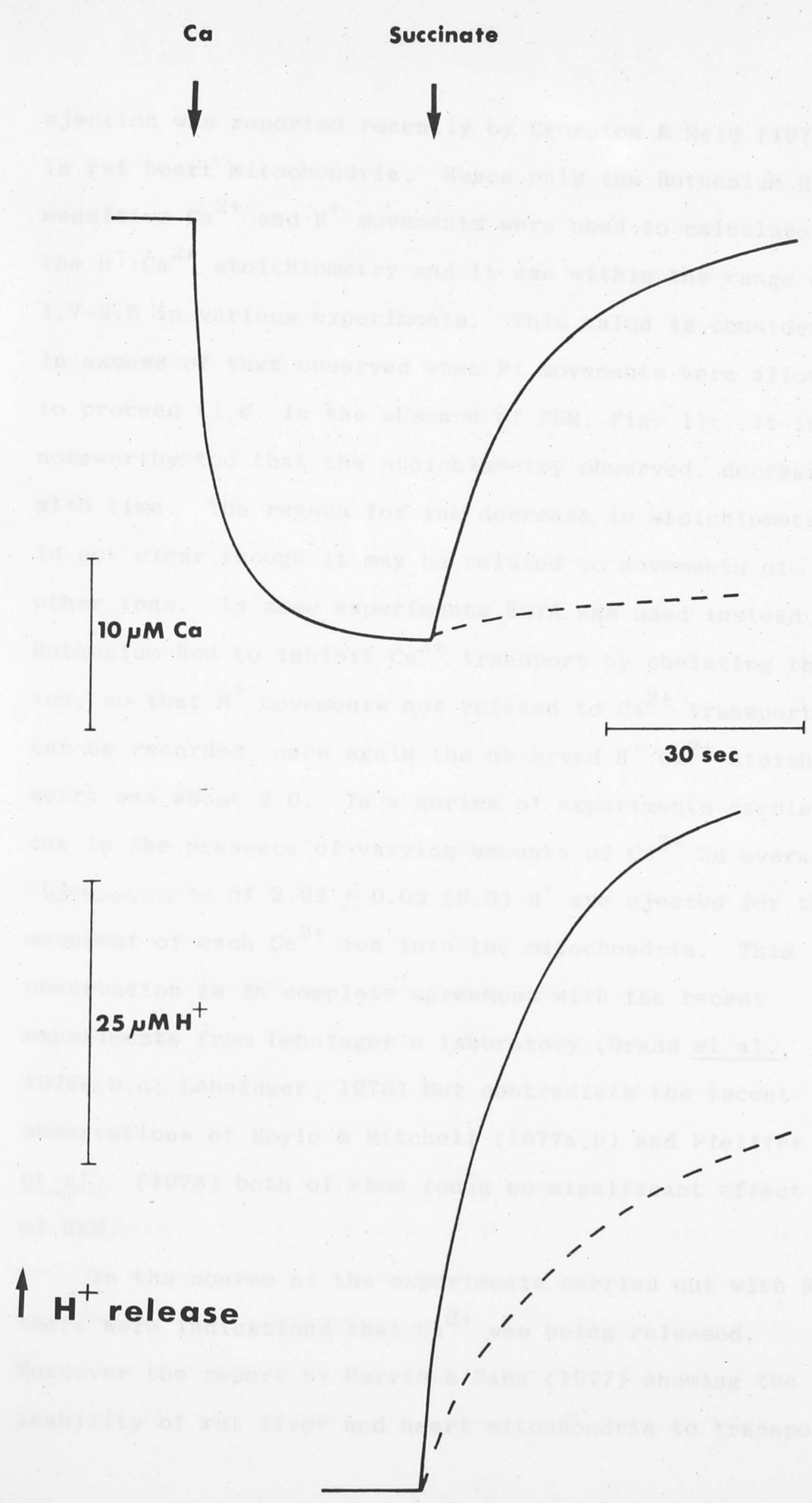

Ca
Succinate
Ca
25 μM Ca or H+
H+
60 sec
H+ release

Fig. 2. Effect of NEM on H^+:Ca^{2+} stoichiometry

The experimental conditions were identical to that described in Fig. 1 except the medium contained 40 μM-NEM also.

Continuous line - Ruthenium Red absent. Discontinuous line - 200 nM-Ruthenium Red was added just before adding succinate.


Ca
Succinate
10 μM Ca
30 sec
25 μM H+
H+ release

ejection was reported recently by Crompton & Heid (1978) in rat heart mitochondria. Hence only the Ruthenium Red-sensitive Ca^{2+} and H^+ movements were used to calculate the $H^+:Ca^{2+}$ stoichiometry and it was within the range of 1.7-2.3 in various experiments. This value is considerably in excess of that observed when Pi movements were allowed to proceed (i.e. in the absence of NEM, Fig. 1). It is noteworthy too that the stoichiometry observed, decreases with time. The reason for the decrease in stoichiometry is not clear though it may be related to movements of other ions. In some experiments EGTA was used instead of Ruthenium Red to inhibit Ca^{2+} transport by chelating the ion, so that H^+ movements not related to Ca^{2+} transport can be recorded; once again the observed $H^+:Ca^{2+}$ stoichiometry was about 2.0. In a series of experiments carried out in the presence of varying amounts of Ca^{2+} an average of 2.02 ± 0.03 (S.D) H^+ are ejected for the movement of each Ca^{2+} ion into the mitochondria. This observation is in complete agreement with the recent experiments from Lehninger's laboratory (Brand et al., 1976a,b,c; Lehninger, 1978) but contradicts the recent observations of Moyle & Mitchell (1977a,b) and Pfeiffer et al. (1978) both of whom found no significant effect of NEM.

In the course of the experiments carried out with NEM, there were indications that Ca^{2+} was being released. Moreover the report by Harris & Zaba (1977) showing the inability of rat liver and heart mitochondria to transport

any Ca^{2+} in the presence NEM and oligomycin but in the absence of added permeant anions, prompted us to reinvestigate Ca^{2+} movements in the presence of -SH reagents in detail. The rest of this chapter is concerned with this.

Influence of NEM and oligomycin on Ca^{2+} transport by rat liver mitochondria

Data in Fig. 3 show the influence of NEM on long term movements of Ca^{2+} across the inner mitochondrial membrane. Addition of NEM (200 nmol/mg of protein) reduces by about 25% the initial rate of Ca^{2+} transport and by about 35% the maximal uptake. This steady-state was obtained in about 1 min and suggests that when Pi-cycling is restricted, mitochondria have a limited capacity to accumulate Ca^{2+}, a conclusion reached earlier by Brand et al. (1976a) and by Harris & Zaba (1977). Fig. 3 also shows that following the attainment of the steady-state NEM treatment prevented the organelle from retaining the ion; extrusion of Ca^{2+} sets in after about 2 min, so that by about 8 min the NEM-sensitive component comprises some 80% of the control Ca^{2+} transport. The NEM-insensitive component in turn consists of oligomycin-sensitive and oligomycin-insensitive components.

The initial rate of Ca^{2+} transport of the oligomycin-insensitive component is about 50% of that of the control and the steady-state concentration is reached in less than 30 sec after the addition of Ca^{2+} to the mitochondria. This steady-state concentration is approx. 50% of that observed for the control. A loss of Ca^{2+} immediately

Fig. 3. Effect of NEM and oligomycin on Ca^{2+} transport by rat liver mitochondria

Mitochondria (1 mg/ml) were equilibrated aerobically at 15°C for 1 min in 2.0 ml of medium containing 150 mM-KCl, 3 mM-Hepes (pH 7.4 with KOH), 5 mM-succinate and 1 μM-rotenone. 25 μM-$CaCl_2$ containing $^{45}CaCl_2$ (0.5 μCi) was added and Ca^{2+} transport followed as described in the Experimental Section.

Control (○), 200 μM-NEM (●), 200 μM-NEM and 5 μg/mg oligomycin of protein (▲).

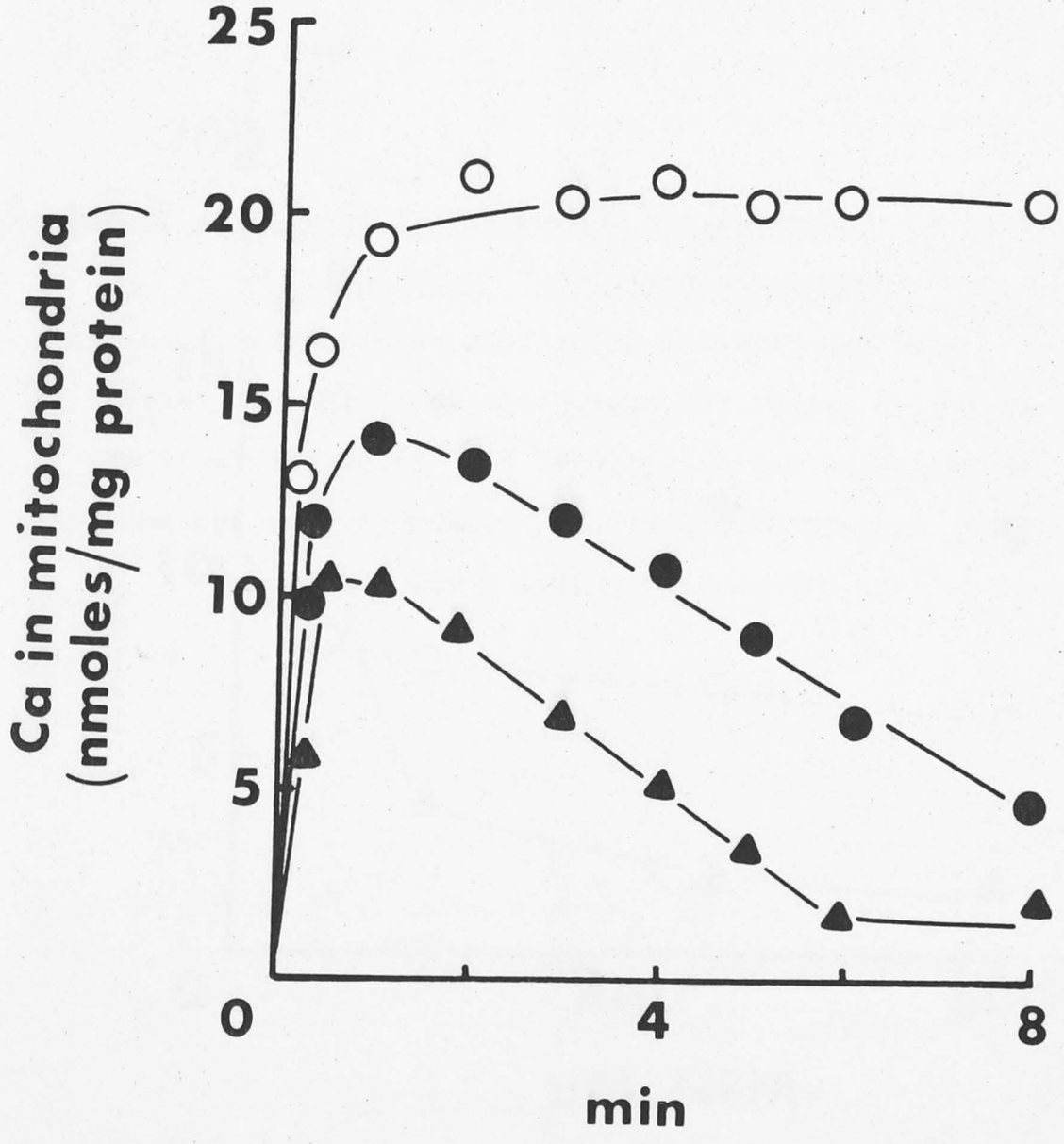
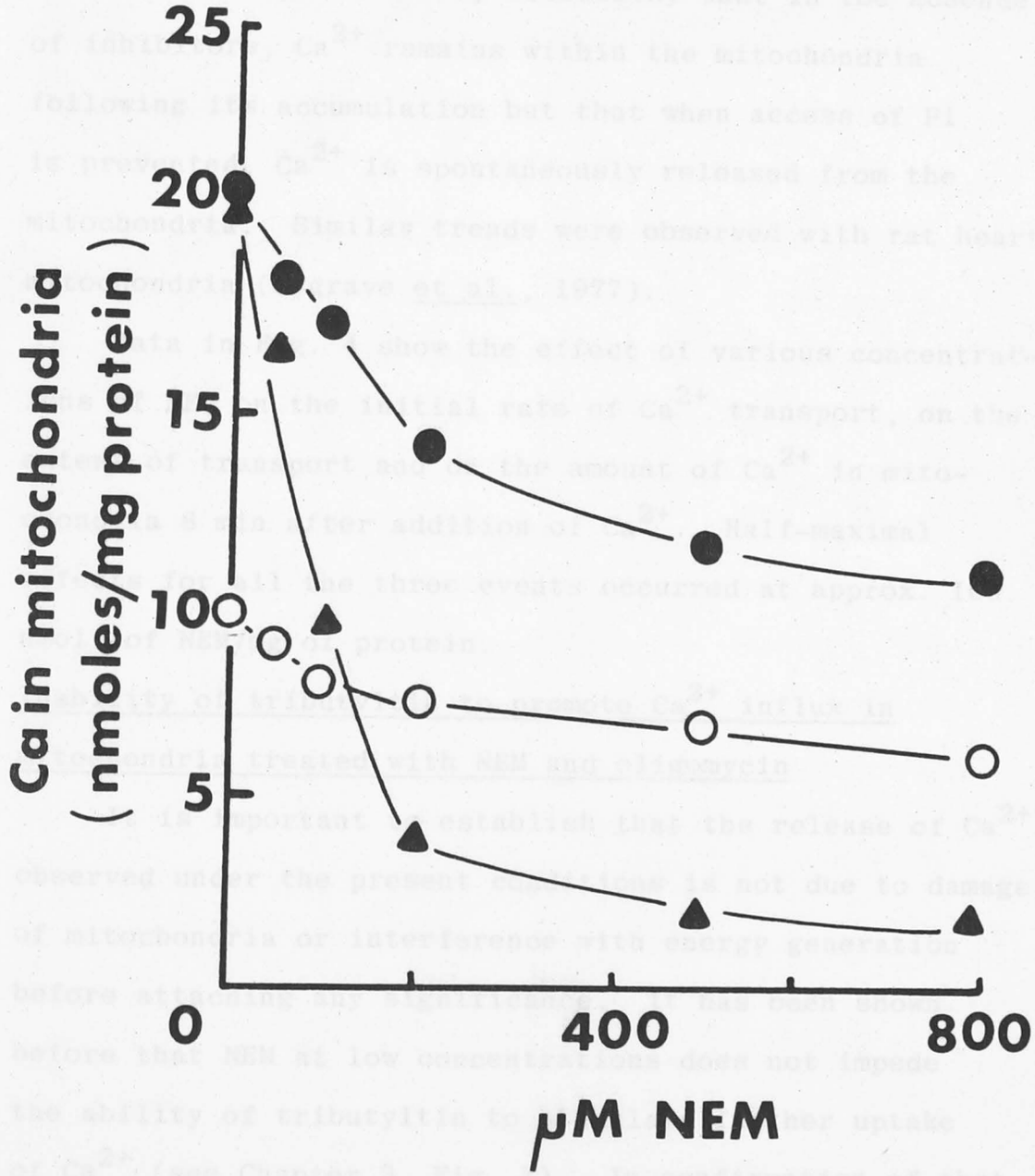

Ca in mitochondria
(nmoles/mg protein)
25
20
15
10
5
0
4
8
min

Fig. 4. Concentration dependence of the effect of NEM on mitochondrial Ca^{2+} transport

The experimental conditions were identical to that in Fig. 3 except the concentration of NEM was varied as indicated. Initial rate of Ca^{2+} transport in 10 sec (○), maximum extent of Ca^{2+} transport (●) and amount of Ca^{2+} in mitochondria after 8 min (▲).


25
20
15
10
5
0
400
800
Ca in mitochondria
(nmoles/mg protein)
μM NEM

ensues so that 6 min after Ca^{2+} addition, the oligomycin-insensitive component is reduced to about 10% of the control Ca^{2+} transport.

It seems particularly noteworthy that in the absence of inhibitors, Ca^{2+} remains within the mitochondria following its accumulation but that when access of Pi is prevented, Ca^{2+} is spontaneously released from the mitochondria. Similar trends were observed with rat heart mitochondria (Bygrave et al., 1977).

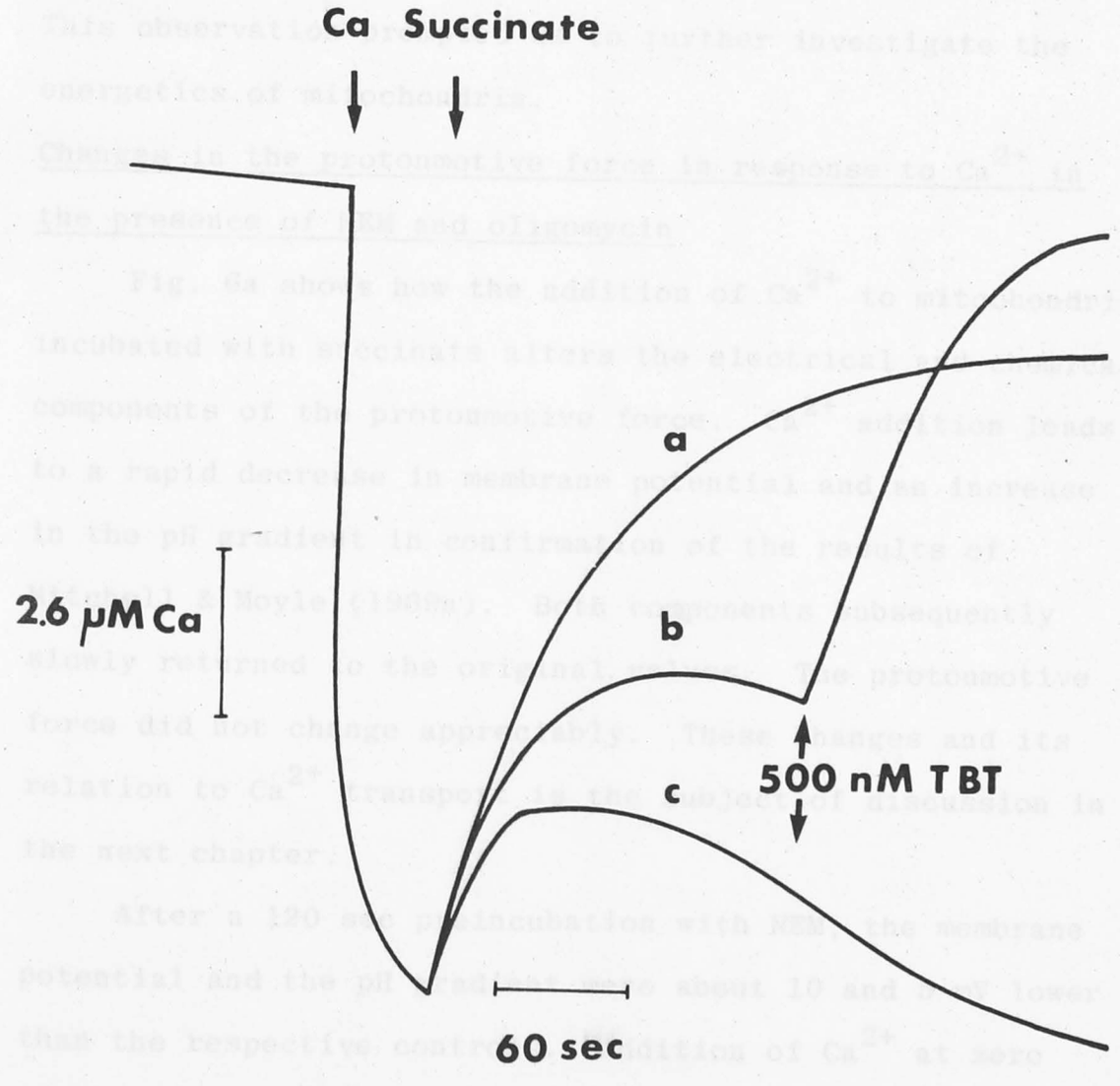
Data in Fig. 4 show the effect of various concentrations of NEM on the initial rate of Ca^{2+} transport, on the extent of transport and on the amount of Ca^{2+} in mitochondria 8 min after addition of Ca^{2+}. Half-maximal effects for all the three events occurred at approx. 100 nmol. of NEM/mg of protein.

Inability of tributyltin to promote Ca^{2+} influx in mitochondria treated with NEM and oligomycin

It is important to establish that the release of Ca^{2+} observed under the present conditions is not due to damage of mitochondria or interference with energy generation before attaching any significance. It has been shown before that NEM at low concentrations does not impede the ability of tributyltin to stimulate further uptake of Ca^{2+} (see Chapter 3, Fig. 7). In confirmation of that observation addition of tributyltin to NEM-treated mitochondria promotes a rapid and immediate stimulation of Ca^{2+} influx (Fig. 5). However, tributyltin fails to promote Ca^{2+} influx when oligomycin is also present along with NEM (lower curve). Subsequent addition of acetate

Fig. 5. Ca^{2+} electrode traces showing the effect of tributyltin on Ca^{2+} transport by liver mitochondria in the presence and absence of NEM and oligomycin

Incubation conditions were as described in Fig. 1. TBT was added where indicated at 500 nM. The temperature was 25°C. Curve a: control (no other additions). Curve b: NEM (200 µM) added before Ca^{2+}. Curve c: NEM plus oligomycin (5 µg/mg protein) added before Ca^{2+}.


Ca Succinate
a
b
c
2.6 μM Ca
500 nM TBT
60 sec

or its addition before that of tributyltin (data not shown) also fails to promote Ca^{2+} transport in the presence of NEM and oligomycin. The pH electrode trace from the same experiment (data not shown) indicated that the ability of tributyltin to mediate Cl^-/OH^- exchange is altered little by the presence of NEM and/or oligomycin. This observation prompted us to further investigate the energetics of mitochondria.

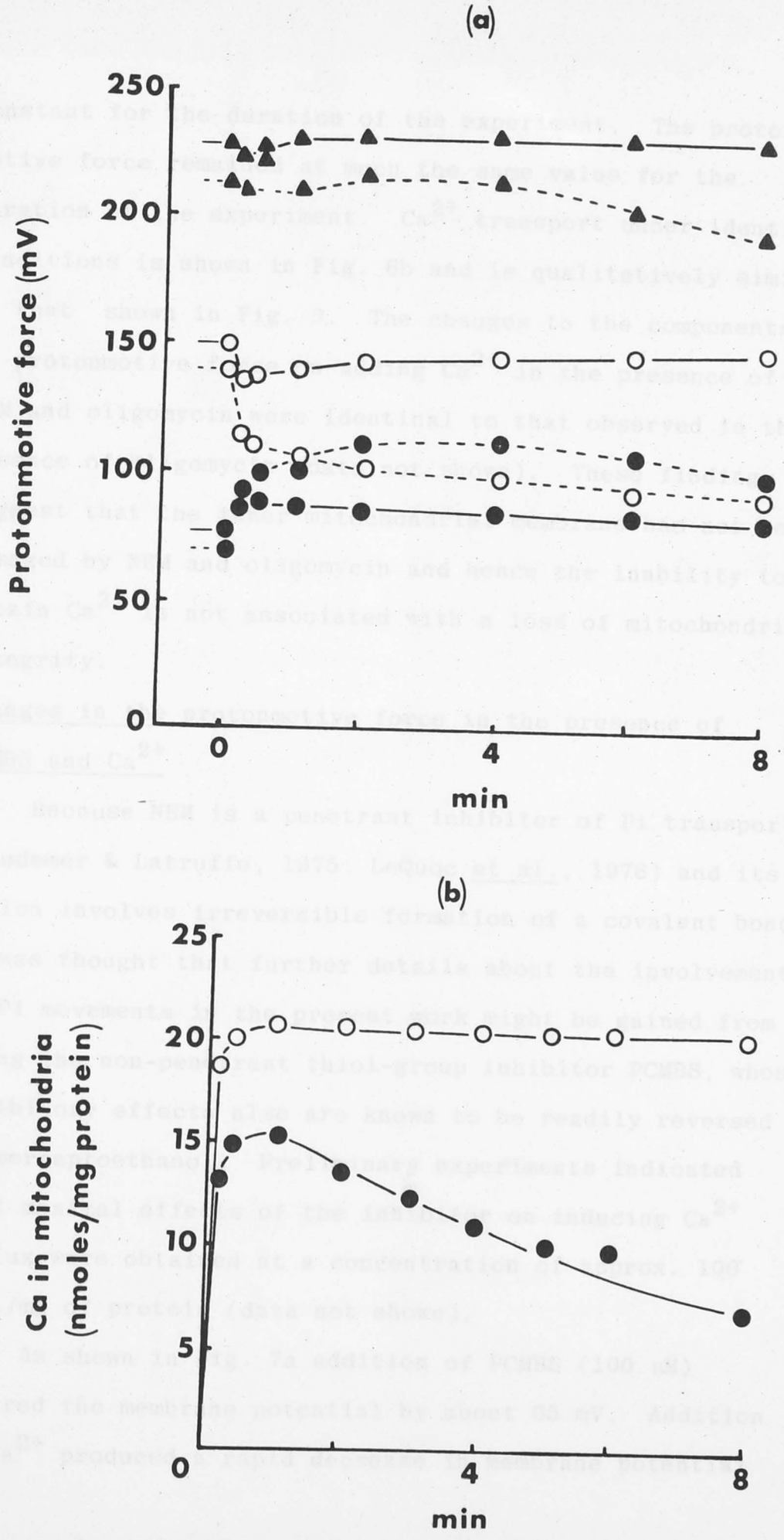
Changes in the protonmotive force in response to Ca^{2+} in the presence of NEM and oligomycin

Fig. 6a shows how the addition of Ca^{2+} to mitochondria incubated with succinate alters the electrical and chemical components of the protonmotive force. Ca^{2+} addition leads to a rapid decrease in membrane potential and an increase in the pH gradient in confirmation of the results of Mitchell & Moyle (1969a). Both components subsequently slowly returned to the original values. The protonmotive force did not change appreciably. These changes and its relation to Ca^{2+} transport is the subject of discussion in the next chapter.

After a 120 sec preincubation with NEM, the membrane potential and the pH gradient were about 10 and 5 mV lower than the respective controls. Addition of Ca^{2+} at zero time produced a rapid decrease in membrane potential the change being about twice that of the control. The membrane potential then remained at the new value without showing any sign of returning to the original value. The pH gradient also increased on addition of Ca^{2+} and the increase was approx. twice that of the control and then remained

Fig. 6. Effect of NEM and Ca^{2+} on the protonmotive force

Mitochondria (1 mg/ml) were equilibrated aerobically at 25°C for 2 min in 4.0 ml of medium containing 150 mM-LiCl, 0.5 mM-Hepes, 5 mM-succinate, 10 µM-^{86}RbCl, 50 µM-[^{14}C]methylamine, 50 µM-sodium [^{3}H]-acetate, 1 µM-rotenone and 0.5 µM-valinomycin. At zero time 25 µM-$CaCl_2$ was added and at appropriate intervals samples were filtered. The filter papers were dissolved in the scintillation fluid and the protonmotive force (a) was calculated as described in the Experimental section. (b) Ca^{2+} transport was followed under identical conditions, but in the absence of radiochemicals, by using $^{45}Ca^{2+}$ (see the Experimental section), except that KCl in the quench mixture was replaced by LiCl. (a) ——, Control; ----, +200 µM-NEM; ▲, protonmotive force; O, membrane potential; ●, pH gradient. (b) O, Control; ●, +200 µm-N-ethyl-maleimide.


(a)
250
200
150
100
50
0
Protonmotive force (mV)
0
4
8
min
(b)
25
20
15
10
5
0
Ca in mitochondria (nmoles/mg protein)
0
4
8
min

constant for the duration of the experiment. The protonmotive force remained at much the same value for the duration of the experiment. Ca^{2+} transport under identical conditions is shown in Fig. 6b and is qualitatively similar to that shown in Fig. 3. The changes to the components of protonmotive force on adding Ca^{2+} in the presence of NEM and oligomycin were identical to that observed in the absence of oligomycin (data not shown). These findings suggest that the inner mitochondrial membrane has not been damaged by NEM and oligomycin and hence the inability to retain Ca^{2+} is not associated with a loss of mitochondrial integrity.

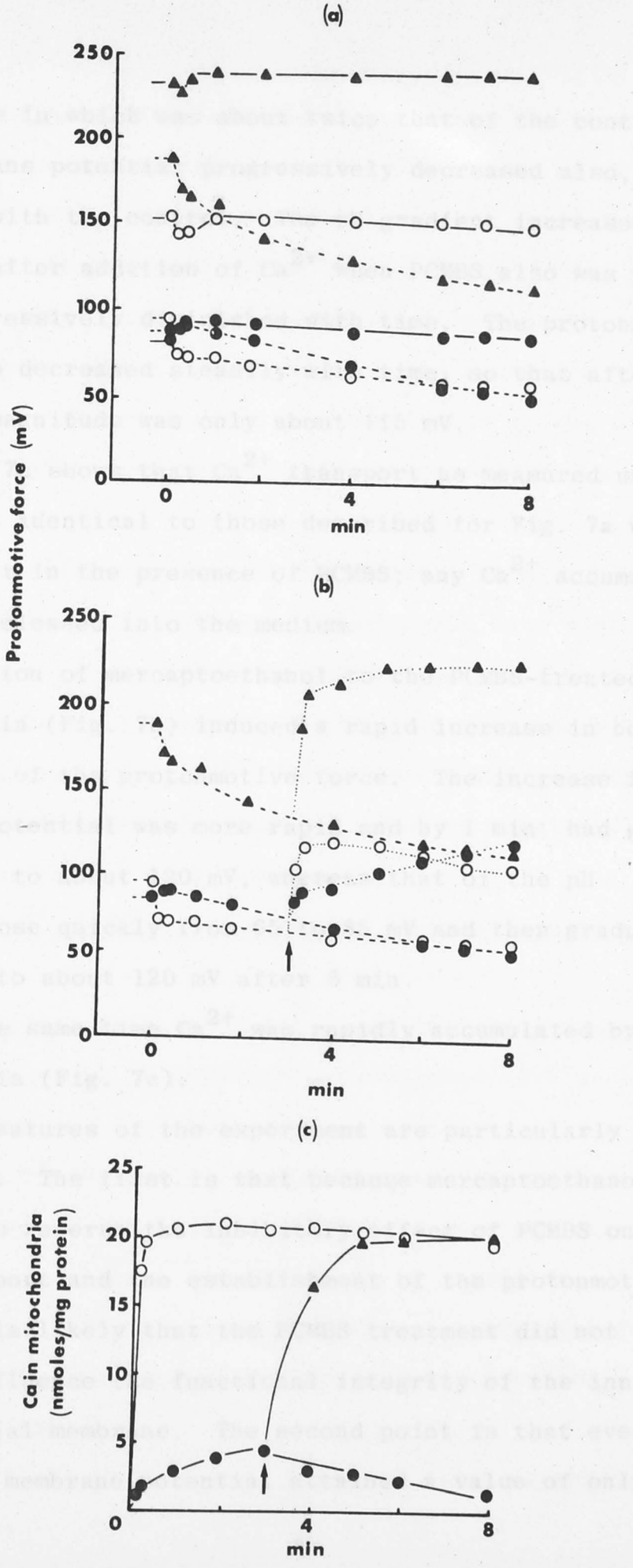
Changes in the protonmotive force in the presence of PCMBS and Ca^{2+}

Because NEM is a penetrant inhibitor of Pi transport (Gaudemer & Latruffe, 1975; LeQuoc et al., 1976) and its action involves irreversible formation of a covalent bond, it was thought that further details about the involvement of Pi movements in the present work might be gained from using the non-penetrant thiol-group inhibitor PCMBS, whose inhibitory effects also are known to be readily reversed by mercaptoethanol. Preliminary experiments indicated that maximal effects of the inhibitor on inducing Ca^{2+} efflux were obtained at a concentration of approx. 100 nmol/mg of protein (data not shown).

As shown in Fig. 7a addition of PCMBS (100 μM) lowered the membrane potential by about 65 mV. Addition of Ca^{2+} produced a rapid decrease in membrane potential

Fig. 7. Effect of PCMBS on the protonmotive force

Incubation conditions were identical with those described in Fig. 6 (a)——, Control; ----, 100 μM-PCMBS; ▲, protonmotive force; ○, membrane potential; ●, pH gradient. (b) ----, +100 μM-PCMBS; ····, after the addition (arrowed) of 1 mM-mercaptoethanol; ▲, protonmotive force; ○, membrane potential; ●, pH gradient. (c) ○, Control; ●, +100 μM-PCMBS; ▲, after adding 1 mM-mercaptoethanol at arrow.


(a)
250
200
150
100
50
0
Protonmotive force (mV)
0
4
8
min
(b)
250
200
150
100
50
0
0
4
8
min
(c)
25
20
15
10
5
0
Ca in mitochondria
(nmoles/mg protein)
0
4
8
min

the change in which was about twice that of the control. The membrane potential progressively decreased also, by contrast with the control. The pH gradient increased only slightly after addition of Ca^{2+} when PCMBS also was present; this progressively diminished with time. The protonmotive force also decreased steadily with time, so that after 8 min its magnitude was only about 115 mV.

Fig. 7c shows that Ca^{2+} transport as measured under conditions identical to those described for Fig. 7a was only slight in the presence of PCMBS; any Ca^{2+} accumulated was soon released into the medium.

Addition of mercaptoethanol to the PCMBS-treated mitochondria (Fig. 7b) induced a rapid increase in both components of the protonmotive force. The increase in membrane potential was more rapid and by 1 min had gone from 75 mV to about 120 mV, whereas that of the pH gradient rose quickly from 65 to 85 mV and then gradually increased to about 120 mV after 5 min.

At the same time Ca^{2+} was rapidly accumulated by the mitochondria (Fig. 7c).

Two features of the experiment are particularly noteworthy. The first is that because mercaptoethanol was able to reverse the inhibitory effect of PCMBS on Ca^{2+} transport and the establishment of the protonmotive force, it is likely that the PCMBS treatment did not greatly influence the functional integrity of the inner mitochondrial membrane. The second point is that even though the membrane potential attained a value of only

about 120 mV after the addition of mercaptoethanol (i.e. some 30 mV lower than the control), the amount of Ca^{2+} accumulated (Fig. 7c) was about the same as that of the control and was retained by the mitochondria. This raises the possibility that the membrane potential is not the rate-limiting factor in Ca^{2+} accumulation and retention by the mitochondria, at least under the conditions of the present experiment. This argument will be discussed in the ensuing chapters.

Alternatively because PCMBS inhibits both Pi/OH^- and Pi/dicarboxylate exchange, PCMBS could have exerted at least part of its effect by inhibiting the transport of succinate. This is supported by the low membrane potential observed before adding Ca^{2+}. Experiments designed to distinguish between the effect of PCMBS on substrate uptake and on Pi transport were unsuccessful.

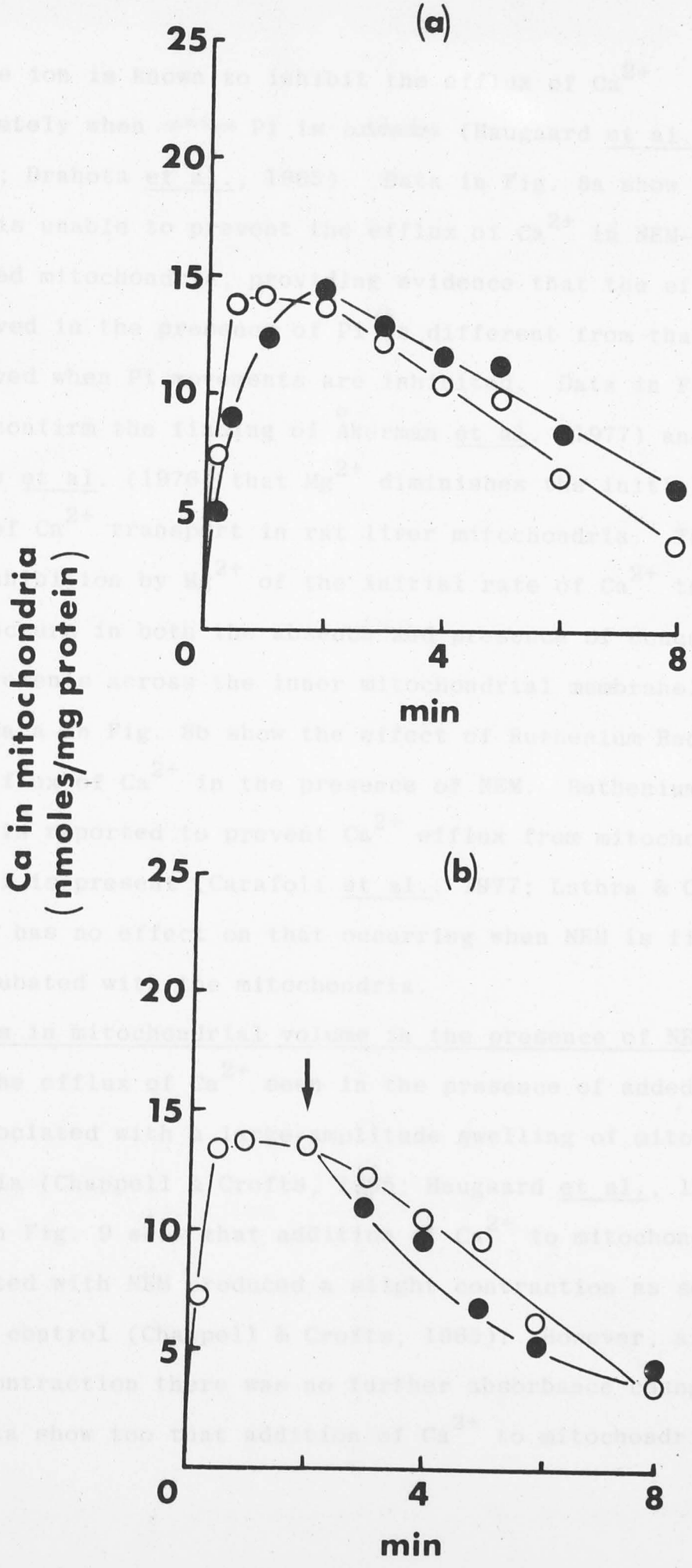
Effect of Mg^{2+} and Ruthenium Red on the NEM-induced efflux of Ca^{2+}

Because the release of Ca^{2+} from the mitochondria in the presence of Pi is invariably associated with uncoupling of the organelle, i.e. increased permeability of the inner membrane to H^+ (Mitchell, 1966), whereas the release of Ca^{2+} observed in the presence of NEM appears not due to the loss of functional integrity of the organelle, it was important to find out whether agents inhibiting Ca^{2+} release in the presence of Pi were effective in the presence of NEM.

The effects of Mg^{2+} were examined first especially

Fig. 8. Effect of Mg^{2+} and Ruthenium Red on the efflux of Ca^{2+} induced by NEM

The reaction media were as described in Fig. 3: (a) 200 μM-N-ethylmaleimide in the absence (O) or presence (●) of 2 mM-$MgCl_2$; (b) 200 μM-NEM present (O), and Ruthenium Red (200 pmol/mg of protein) was added at the point indicated with the arrow (●).


(a)
25
20
15
10
5
0
4
8
min
Ca in mitochondria
(nmoles/mg protein)
(b)
25
20
15
10
5
0
4
8
min

as the ion is known to inhibit the efflux of Ca^{2+} completely when Pi is added (Haugaard et al., 1969a; Drahota et al., 1965). Data in Fig. 8a show that Mg^{2+} is unable to prevent the efflux of Ca^{2+} in NEM-treated mitochondria, providing evidence that the efflux observed in the presence of Pi is different from that observed when Pi movements are inhibited. Data in Fig. 8a also confirm the finding of Åkerman et al. (1977) and of Hutson et al. (1976) that Mg^{2+} diminishes the initial rate of Ca^{2+} transport in rat liver mitochondria. Thus the inhibition by Mg^{2+} of the initial rate of Ca^{2+} transport occurs in both the absence and presence of concomitant Pi movements across the inner mitochondrial membrane.

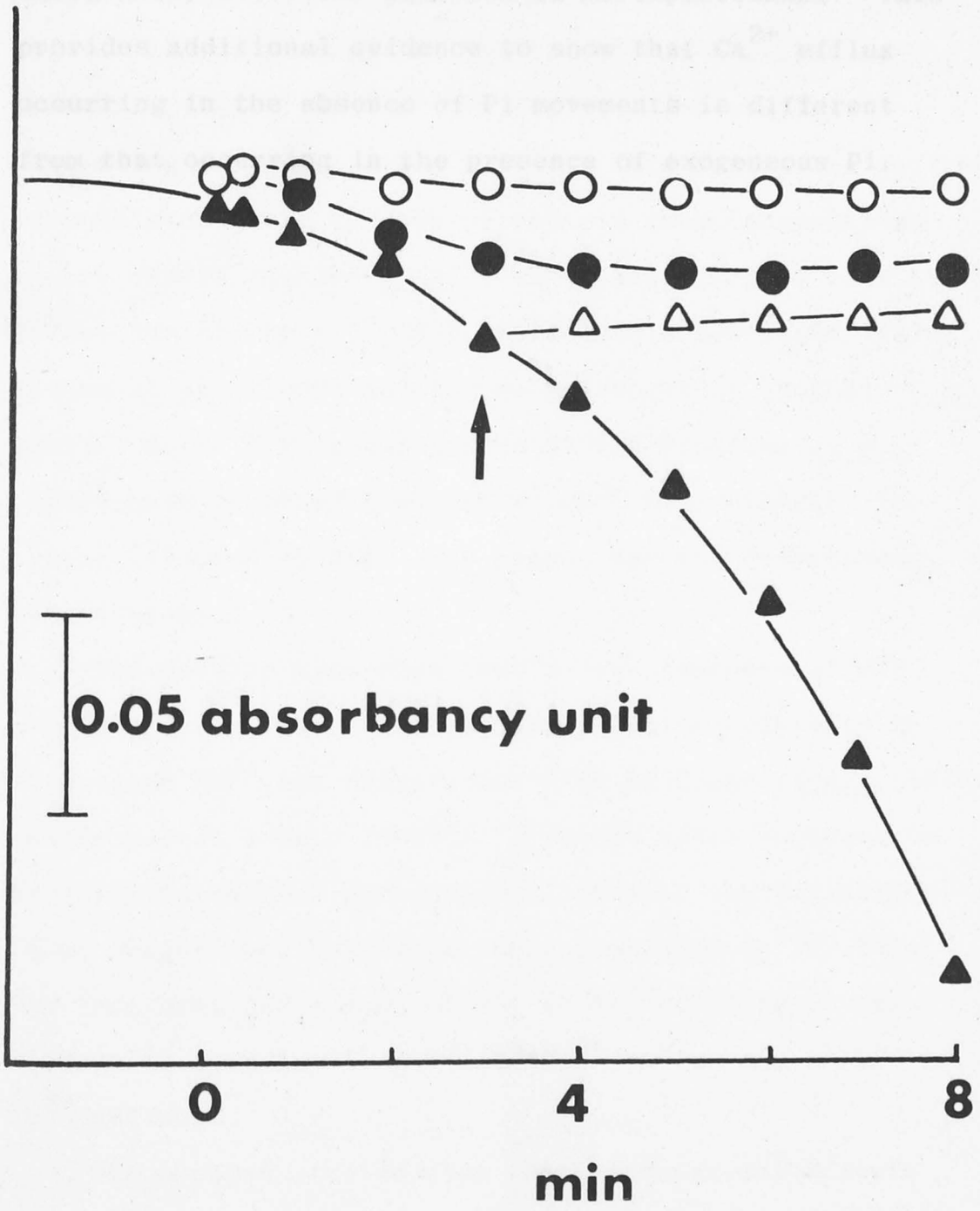
Data in Fig. 8b show the effect of Ruthenium Red on the efflux of Ca^{2+} in the presence of NEM. Ruthenium Red, which is reported to prevent Ca^{2+} efflux from mitochondria when Pi is present (Carafoli et al., 1977; Luthra & Olson, 1977), has no effect on that occurring when NEM is first preincubated with the mitochondria.

Changes in mitochondrial volume in the presence of NEM

The efflux of Ca^{2+} seen in the presence of added Pi is associated with a large-amplitude swelling of mitochondria (Chappell & Crofts, 1965; Haugaard et al., 1969a). Data in Fig. 9 show that addition of Ca^{2+} to mitochondria incubated with NEM produced a slight contraction as seen in the control (Chappell & Crofts, 1965). However, after such contraction there was no further absorbance change. The data show too that addition of Ca^{2+} to mitochondria

Fig. 9. Volume changes of mitochondria during Ca^{2+} transport in the absence and presence of NEM or PCMBS

Conditions were as in Fig. 3, except that the reaction was started with 0.5 μM-valinomycin. After 2 min incubation, 25 μM-$CaCl_2$ was added. ○, Control; ●, +200 μM-NEM; ▲, +100 μM-PCMBS: at the point indicated by the arrow, 1 mM-mercaptoethanol was also added (△).


0.05 absorbancy unit
0
4
8
min

treated with PCMBS resulted in a slight swelling which, although it increases gradually with time, was arrested immediately after the addition of mercaptoethanol. This provides additional evidence to show that Ca^{2+} efflux occurring in the absence of Pi movements is different from that occurring in the presence of exogeneous Pi.

DISCUSSION

The results shown in this chapter confirm the H^+:Ca^{2+} stoichiometry of 1.0 obtained normally (Lehninger *et al.*, 1967). In the presence of NEM to inhibit endogeneous Pi movements, the H^+:Ca^{2+} stoichiometry increases to 2.0 suggesting a H^+:site ratio of 4.0. This finding is entirely consistent with the recent reports from Lehninger's laboratory (Alexandre *et al.*, 1978; Reynafarje & Lehninger, 1978b; Vercesi *et al.*, 1978; Villalobo & Lehninger, 1979; Fiskum *et al.*, 1979) and Azzone's laboratory (Pozzan *et al.*, 1979). Moyle & Mitchell (1977a,b) and Pfeiffer *et al.* (1978) have reported that the H^+:Ca^{2+} stoichiometry is little affected by NEM. The reason for the discrepancy is not clear.

The results also show that in the presence of NEM and oligomycin, mitochondria have a limited capacity to accumulate Ca^{2+}, an observation made by Brand *et al.* (1976a) and by Harris & Zaba (1977). A second major consequence of NEM treatment of mitochondria, is that the organelle is no longer able to retain the accumulated Ca^{2+}. Thus NEM treatment has a dual effect on Ca^{2+}-cycling in rat liver mitochondria; it prevents Ca^{2+} influx and promotes Ca^{2+} efflux.

The present studies show that near-maximal effects of NEM on Ca^{2+}-cycling were obtained at approx. 150 nmol/mg of protein. This value may be compared with that of approx. 90 nmol/mg of protein obtained by Hutson (1977),

who examined the effect of NEM on steady-state kinetics of energy-dependent Ca^{2+} transport in rat liver mitochondria. Haugaard et al. (1969b) studied the effect of 5,5'-dithiobis-(2-nitrobenzoic acid) on Ca^{2+} transport in rat liver mitochondria and observed that near-maximal inhibition of influx occurred at a concentration of approx. 50 nmol/mg of protein. No effect of the thiol-group inhibitor was seen on Ca^{2+} efflux, presumably because of the inclusion in the reaction medium of ATP, which itself would induce Ca^{2+} retention by mitochondria. According to these workers, NEM produced effects similar to those of 5,5'-dithiobis-(2-nitrobenzoic acid).

Anion transport systems whose activities influence Ca^{2+} retention by mitochondria include the Pi and adenine nucleotide translocation systems (Bygrave, 1978a); each is sensitive to thiol-group inhibitors. In the present experiments the activities of each should have been minimal, since the concentration of NEM required to inhibit Pi transport via the Pi/OH^- exchange is in the range 30 nmol/mg of protein (Coty & Pedersen, 1974) to approx. 90 nmol/mg of protein (Klingenberg et al., 1974). Approx. 60 nmol/mg of protein is required to inhibit the Pi/Pi exchange when butylmalonate also is present to inhibit the Pi/dicarboxylate exchange (Meijer et al., 1970) and near-maximal inhibition of adenine nucleotide translocation occurs at NEM concentrations of 30 nmol/mg of protein (Leblanc & Clauser, 1972; Vignais & Vignais, 1972). The NEM-induced efflux of Ca^{2+} cannot be attributed on the basis

of the present experiments solely to an action of the inhibitor on Pi and adenine nucleotide movements, but some assessment of the likelihood can be made.

Studies by Sabadie-Pialoux & Gautheron (1971) indicate that the number of free thiol groups in rat liver mitochondria approach 90 nmol/mg of protein, but this number can vary according to the experimental conditions used. Addition of ADP plus Pi to well coupled mitochondria for instance, leads to an increase by some 30% of free thiol groups (Sabadie-Pialoux & Gautheron, 1971). This could reflect a conformational change in the inner mitochondrial membrane that is NEM-sensitive (Leblanc & Clauser, 1972; Vignais & Vignais, 1972). Other conditions under which the reactivity of thiol groups in mitochondria is altered include the anionic composition of the reaction medium, the pH and the metabolic status of the mitochondria (see Scott et al., 1970; Klingenberg et al., 1974). Moreover, evidence exists that in addition to reacting with thiol groups, NEM reacts with side-chain amino groups that contain active hydrogens (Brewer & Riehm, 1967).

Thus although in the first instance it would seem unlikely that inhibition of Pi transport alone contributes to the induced Ca^{2+} efflux, the possibility may not be so unrealistic when consideration to the above is given. It is worth recalling also that the interaction of Ca^{2+} with rat liver mitochondria produces changes in the microscopic appearance of the organelle (Greenawalt et al., 1964), which also might lead to unmasking of thiol groups.

Inhibition by NEM of succinate oxidation, and therefore the driving force for Ca^{2+} transport seems unlikely on two grounds; first, the protonmotive force is only slightly decreased in the presence of the compound (Fig. 6) and, second, addition of tributyltin to NEM-treated mitochondria, induces an immediate influx of Ca^{2+} into mitochondria (Fig. 5). It has been suggested that specific NEM-sensitive groups are located close to the Ca^{2+} carrier and the data do not rule out this possibility. On the other hand, inability of tributyltin to induce Ca^{2+} influx in the presence of NEM and oligomycin (Fig. 5) is not due to an altered protonmotive force, indicating either the combination of NEM and oligomycin causes an inhibition of the Ca^{2+} carrier itself or an absolute dependence on Pi for Ca^{2+} transport.

Previous studies in many laboratories have shown that efflux of Ca^{2+} from mitochondria occurs as a result of 'damage' to the mitochondrial inner membrane. Such efflux is associated, for example, with swelling of mitochondria, an increase in ATPase activity, and concomitant loss of ability of the mitochondria to carry out oxidative phosphorylation (see Saris, 1963; Bygrave & Reed, 1970). These events, which are considerably enhanced by the presence of Pi, are attributable to the uncoupling effect of Ca^{2+} on mitochondrial energy-linked functions originally observed by Lehninger (1949).

For this reason it was important to assess the 'integrity' of the mitochondria after Ca^{2+} efflux under

the present experimental conditions. The experiments revealed (Figs. 6,7 & 9) that swelling of the organelle does not accompany Ca^{2+} release, nor does the protonmotive force change to an extent that reflects a complete loss of impermeability to H^+, a finding that would produce evidence of membrane 'damage'. Indeed, the almost complete reversibility of the system in terms of swelling, Ca^{2+} transport and the protonmotive force were clearly demonstrable with the appropriate additions of PCMBS and mercaptoethanol.

Further evidence that the mechanism of Ca^{2+} efflux seen here in the presence of NEM differs from that occurring in the presence of Pi was the finding that Mg^{2+} and Ruthenium Red, each of which inhibits Ca^{2+} efflux in the presence of Pi (Haugaard et al., 1969a; Carafoli et al., 1977; Luthra & Olson, 1977) were ineffective in preventing Ca^{2+} efflux under the present incubation conditions. Moreover in vivo administration of glucagon, which can induce retention of Ca^{2+} in the presence of Pi in subsequently isolated mitochondria, was ineffective in inducing retention in the presence of NEM (Prpic et al., 1978).

The assemblage of these data thus shows that the mechanism of efflux of Ca^{2+} from NEM-treated mitochondria is distinctly different from that seen in the presence of Pi-cycling. This finding, certainly emphasizes the important and possibly vital physiological role played by thiol groups in the movement of Ca^{2+} across the inner mitochondrial membrane, especially with regard to their

interaction with the Ca^{2+}-translocation cycle located in that membrane.

After this work was completed Lofrumento & Zanotti (1978) and Pfeiffer et al. (1978) reported that NEM can induce efflux of Ca^{2+} from rat liver mitochondria.

CHAPTER 5

PROTONMOTIVE FORCE AND MITOCHONDRIAL CALCIUM TRANSPORT

PROTONMOTIVE FORCE AND MITOCHONDRIAL CALCIUM TRANSPORT

INTRODUCTION

Electron transport by the respiratory chain results in a charge separation i.e. ejection of H^+ on the cytosolic side leaving behind OH^- ions on the matrix side of the inner mitochondrial membrane. This charge separation generates the protonmotive force which is equivalent to the high-energy state or intermediate, coupling oxidation, phosphorylation and ion transport as advocated by Mitchell (see Mitchell, 1979 for a recent appraisal of chemiosmosis). The protonmotive force has two components, an electrical component (membrane potential) and chemical component, (pH gradient).

The existence of a membrane potential negative in the matrix of the mitochondria and dependent on the metabolic activity of the organelle, has been widely accepted (Rottenberg, 1973,1975), though microelectrode implantation into giant mitochondria failed to detect any metabolism-dependent membrane potential (Tupper & Tedeschi, 1969). The membrane potential recorded by the electrode technique was about 10-20 mV, positive inside. The apparent difficulty associated with the location of the electrode tip in the extremely low inner matrix volume, with extensive folding of the inner membrane was suggested to be responsible for the inability to record any metabolism-dependent membrane potential (for a recent discussion see Rottenberg, 1979; Tedeschi, 1979). On the other hand the membrane potential calculated using

the distribution of K^+ with the K^+-electrode (Mitchell & Moyle, 1969a) or $^{42}K^+$ (Padan & Rottenberg, 1973) or $^{86}Rb^+$ (Nicholls, 1974) in the presence of valinomycin were of the order of 150 mV, negative in the matrix under state 4 conditions. Lower values, of the order of 130 mV, have been reported (Padan & Rottenberg, 1973) perhaps attributable to technical or experimental difficulties. For example Nicholls (1974) pointed out that anaerobiosis associated with sedimenting the mitochondria by centrifugation can decrease the membrane potential by about 50 mV. Skulachev (1972) also measured a membrane potential negative in the matrix using synthetic, lipophilic, highly-permeable cations. Åkerman & Wikstrom (1976) recorded a membrane potential of about 150 mV negative in the matrix, using safranine.

The existence of a pH gradient across the inner membrane has been clearly established. The pH gradient was measured using the distribution of weak acids, internal pH indicators or with pH electrodes after disrupting the mitochondria (see Rottenberg, 1975 for a review). The pH gradient estimated with these techniques conclusively demonstrated that the matrix was alkaline and a pH gradient of about 80 mV i.e. approximately 1.3 pH units, was recorded using acetate distribution in state 4 (Nicholls, 1974). Recent measurements based on the ionization of Pi for measuring pH using ^{31}P nuclear magnetic resonance provide confirmatory evidence for an alkaline matrix pH (see Shulman et al., 1979 for a review).

Energy-dependent accumulation of Ca^{2+} by mitochondria requires the oxidation of substrates by the respiratory chain or the hydrolysis of ATP by F_1-ATPase. The movement of Ca^{2+} into mitochondria was thought to be electrophoretic (Selwyn et al., 1970a; Lehninger, 1974), though very few experiments were directed to confirm this. Mitchell & Moyle (1969a) first showed that addition of a pulse of Ca^{2+} causes a decrease in membrane potential and an increase in pH gradient. Rottenberg & Scarpa (1974) showed that Ca^{2+} distribution under steady-state conditions was in equilibrium with K^+ distribution in the presence of valinomycin when acetate was included to minimise binding. On the other hand, Puskin et al. (1976) and Azzone et al. (1977) showed that the steady-state distribution of divalent cations was lower than that of monovalent cations. They also showed that even among divalent cations the distribution of Mn^{2+} was lower than that for Ca^{2+}. (Although use of Mn^{2+} as a paramagnetic analogue gave true free Mn^{2+} gradients, it must be remembered that Mn^{2+} transport differs significantly from Ca^{2+} transport, see Gunter et al., 1978). This discrepancy in steady-state accumulation ratio was taken as evidence for the existence of an efflux mechanism. Evidence for an efflux mechanism is now accumulating (Carafoli & Crompton, 1978; Lehninger et al., 1978a,b; Nicholls, 1978a,b). Heaton & Nicholls (1976) measured steady-state Ca^{2+} and Rb^+ gradients in the presence of A23187 and valinomycin and showed that Ca^{2+}

entered the mitochondria via a uniport mechanism limited by the activity of the respiratory chain.

In this chapter the results of experiments which were concerned with the relationship between the protonmotive force and Ca^{2+} transport are described. The components of the protonmotive force were perturbed by using protonophore CCCP, anionophore tributyltin, which mediates Cl^-/OH^- exchange in a Cl^- containing medium, and antimycin A, an inhibitor of succinate oxidation. The effect of these agents on mitochondrial Ca^{2+} transport was studied and an attempt was made to correlate changes in membrane potential with altered Ca^{2+} transport.

RESULTS

Ca^{2+} accumulation and its influence on the components of the protonmotive force

Data in Fig. 1 show the effect of addition of 30 μM Ca^{2+} to mitochondria respiring in the presence of succinate on the components of protonmotive force. The accumulation of Ca^{2+} (Fig. 1b) leads to a rapid and immediate decrease in membrane potential and an increase in the pH gradient as was also observed by Mitchell & Moyle (1969a). The earliest time at which a reproducible-change in membrane potential and pH gradient could be measured in these experiments was 10 sec after Ca^{2+} addition. These measurements were greatly facilitated by lowering the incubation temperature to 9°C. The membrane potential and the pH gradient slowly return to their original values; the protonmotive force remains at much the same value throughout. As will be seen below the magnitude of the change in membrane potential and the pH gradient as well as its ability to return to the original value is a function of the added Ca^{2+} concentration. The decrease in membrane potential is interpreted as due to the translocation of ionic Ca^{2+} carrying one or two positive charges (Moyle & Mitchell, 1977a,b,c; Reynafarje & Lehninger, 1977; Lehninger *et al.*, 1978a) and provides further direct evidence for the electrophoretic nature of Ca^{2+} transport by rat liver mitochondria. This conclusion is substantiated by the finding that Ruthenium Red, a potent inhibitor of Ca^{2+} influx into mitochondria (Moore,

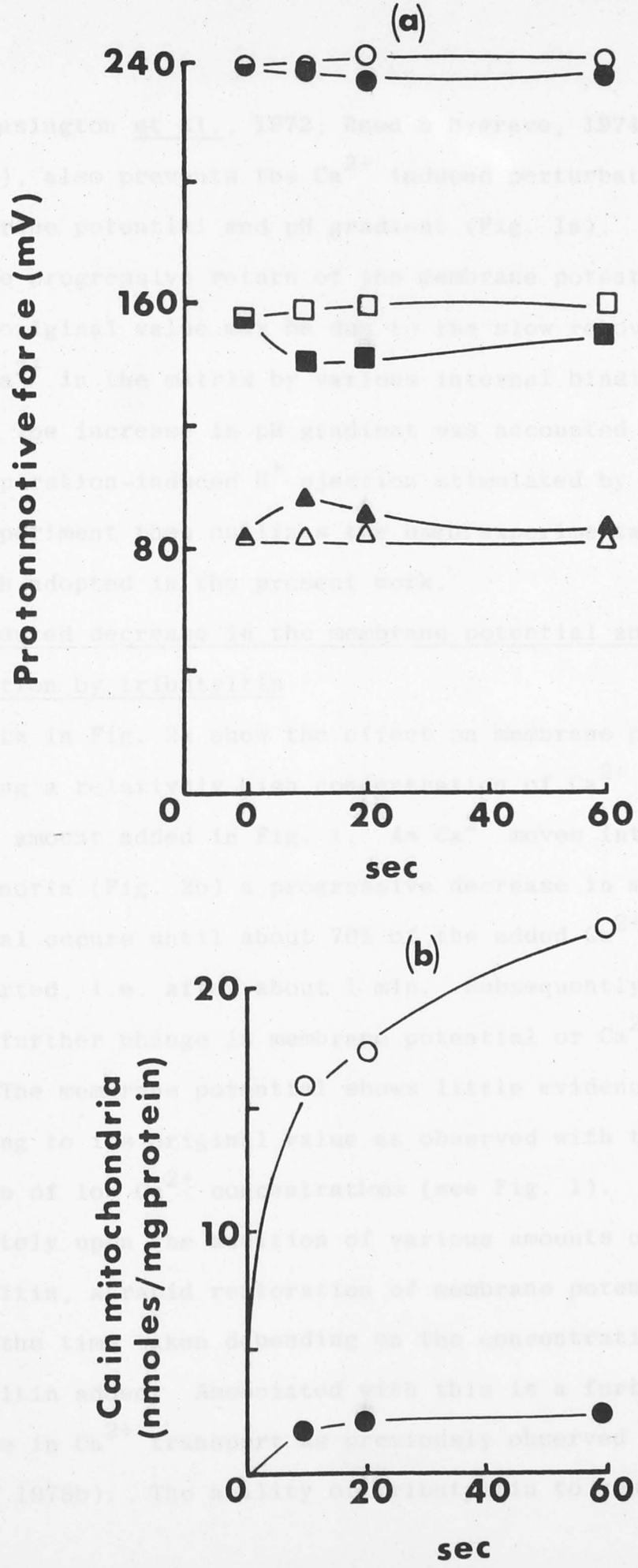
Fig. 1. Effect of Ca^{2+} influx on the components of the protonmotive force

Mitochondria (1 mg/ml) were equilibrated aerobically at 25°C for 2 min in a medium containing 150 mM-LiCl, 0.5 mM-KCl, 3 mM-Hepes (pH to 7.4 with tris at room temperature), 5 mM-sodium succinate 10 µM-^{86}RbCl (0.12 µCi/ml), 50 µM-[^{14}C]methyl ammonium hydrochloride (0.3 µCi/ml), 50 µM-[^{3}H] sodium acetate (1.2 µCi/ml), 1 µM-rotenone, and 0.5 µM-valinomycin. Ruthenium Red was present as indicated at a concentration of 200 pmol/mg of protein. 30 µM-$CaCl_2$ was added at time zero in the figure. At the indicated times, samples were removed for the determination of the components of the protonmotive force as described in the Experimental section. Ca^{2+} transport (Fig. 1b) was followed under identical conditions in the absence (○) or presence (●) of Ruthenium Red using $^{45}Ca^{2+}$ and the centrifugation technique (see the Experimental Section).

Fig. a: Protonmotive force (○ , ●)

Membrane potential (□ , ■)

pH gradient (△ , ▲); open symbols, Ruthenium Red present; closed sybols, Ruthenium Red absent.


(a)
240
160
80
Protonmotive force (mV)
0
0
20
40
60
sec
(b)
20
10
Ca in mitochondria
(nmoles/mg protein)
0
20
40
60
sec

1971; Vasington et al., 1972; Reed & Bygrave, 1974a; Fig. 1b), also prevents the Ca^{2+} induced perturbation of membrane potential and pH gradient (Fig. 1a).

The progressive return of the membrane potential to its original value may be due to the slow removal of ionic Ca^{2+} in the matrix by various internal binding sites. The increase in pH gradient was accounted for by the respiration-induced H^{+} ejection stimulated by Ca^{2+}. This experiment then outlines the basic experimental approach adopted in the present work.

Ca^{2+}-induced decrease in the membrane potential and its restoration by tributyltin

Data in Fig. 2a show the effect on membrane potential of adding a relatively high concentration of Ca^{2+} (100 μM) cf. the amount added in Fig. 1. As Ca^{2+} moves into the mitochondria (Fig. 2b) a progressive decrease in membrane potential occurs until about 70% of the added Ca^{2+} has been transported, i.e. after about 1 min. Subsequently there is little further change in membrane potential or Ca^{2+} transport. The membrane potential shows little evidence of returning to its original value as observed with the addition of low Ca^{2+} concentrations (see Fig. 1). Immediately upon the addition of various amounts of tributyltin, a rapid restoration of membrane potential ensues, the time taken depending on the concentration of tributyltin added. Associated with this is a further increase in Ca^{2+} transport as previously observed (Bygrave et al., 1978b). The ability of tributyltin to restore

Fig. 2. Restoration of Ca^{2+}-induced decrease in membrane potential by tributyltin

Incubation conditions were identical to those described in Fig. 1, except that the temperature was 9°C and the amount of Ca^{2+} added was 100 µM (time zero in the Fig). Varying concentrations of tributyltin were added 1 min after adding Ca^{2+} as indicated in the Fig (arrowed).

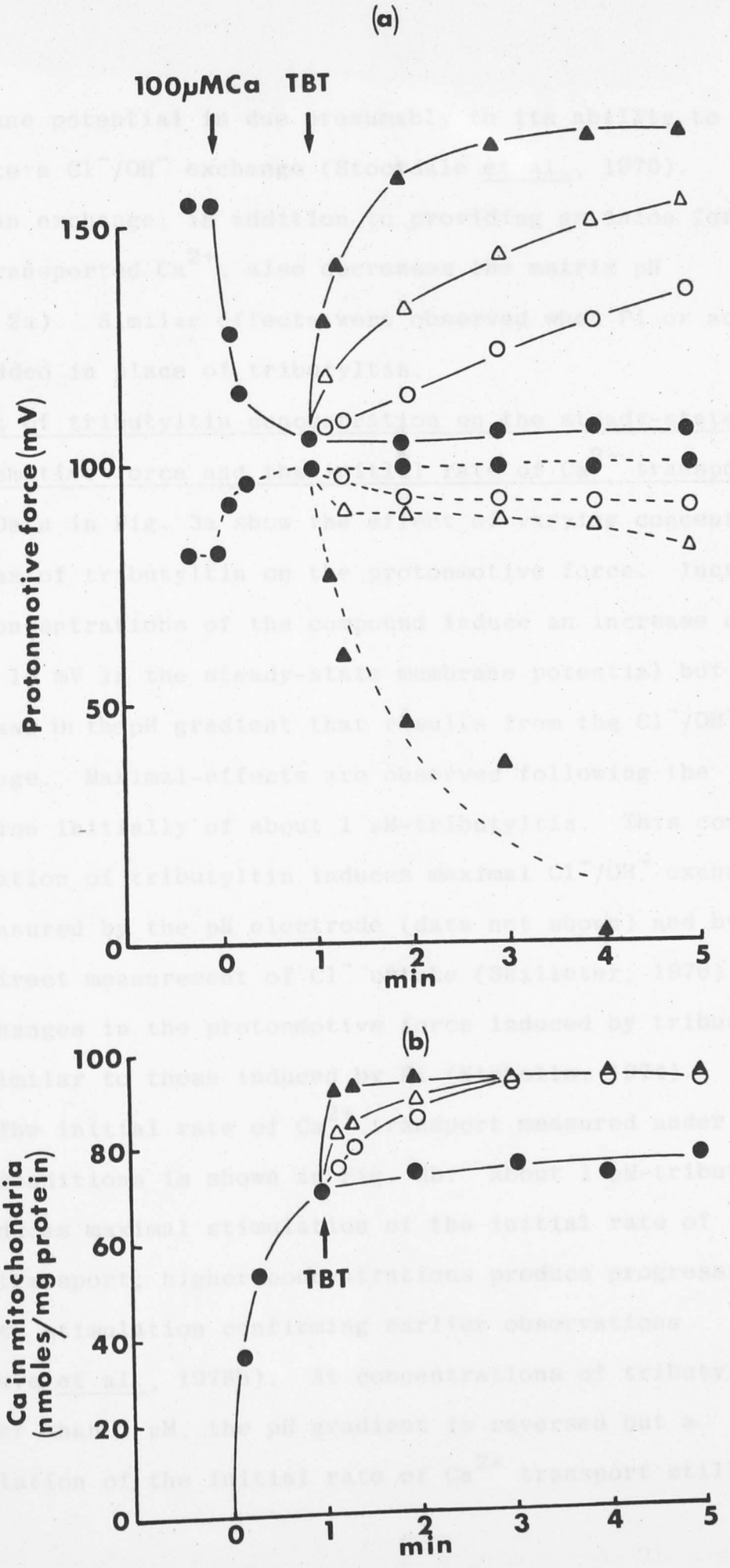
Fig. a: control (●)

after adding 0.25 µM (○), 0.5 µM (△) and 1.0 µM (▲) tributyltin

Membrane potential (continuous line)

pH gradient (broken line)

Fig. b: Ca^{2+} transport in control (●) and after adding 0.25 µM (○), 0.5 µM (△), and 1.0 µM (▲) tributyltin.


(a)
100μMCa
TBT
150
100
50
0
Protonmotive force (mV)
0
1
2
3
4
5
min
(b)
100
80
60
40
20
0
Ca in mitochondria (nmoles/mg protein)
TBT
0
1
2
3
4
5
min

membrane potential is due presumably to its ability to mediate a Cl^-/OH^- exchange (Stockdale et al., 1970). Such an exchange, in addition to providing an anion for the transported Ca^{2+}, also decreases the matrix pH (Fig. 2a). Similar effects were observed when Pi or acetate was added in place of tributyltin.

Effect of tributyltin concentration on the steady-state protonmotive force and the initial rate of Ca^{2+} transport

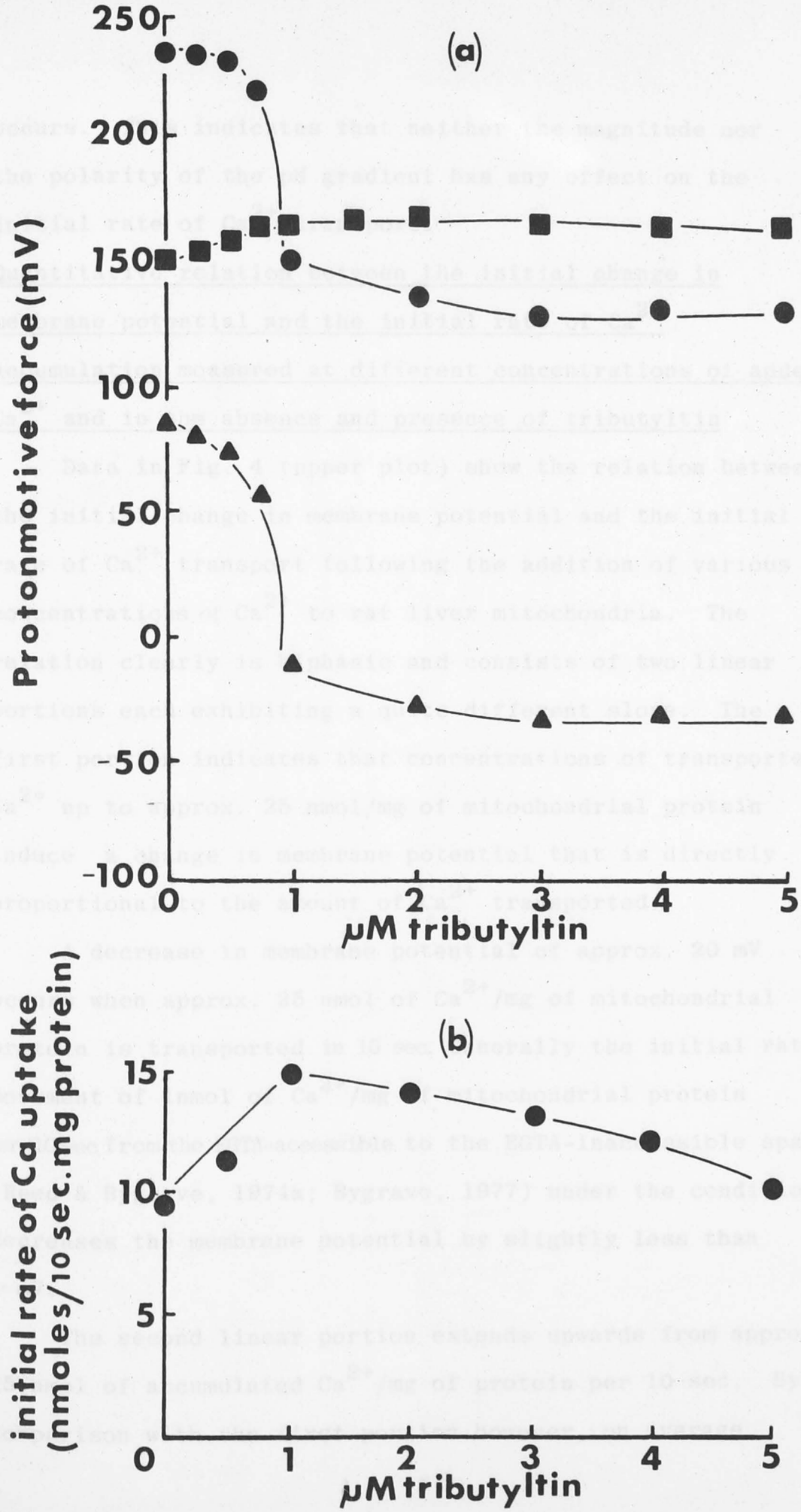
Data in Fig. 3a show the effect of varying concentrations of tributyltin on the protonmotive force. Increasing concentrations of the compound induce an increase of 10 to 15 mV in the steady-state membrane potential but decrease in the pH gradient that results from the Cl^-/OH^- exchange. Maximal-effects are observed following the addition initially of about 1 μM-tributyltin. This concentration of tributyltin induces maximal Cl^-/OH^- exchange as measured by the pH electrode (data not shown) and by the direct measurement of Cl^- uptake (Skilleter, 1976): the changes in the protonmotive force induced by tributyltin are similar to those induced by Pi (Nicholls, 1974).

The initial rate of Ca^{2+} transport measured under the same conditions is shown in Fig. 3b. About 1 μM-tributyltin induces maximal stimulation of the initial rate of Ca^{2+} transport; higher concentrations produce progressively less stimulation confirming earlier observations (Bygrave et al., 1978b). At concentrations of tributyltin greater than 1 μM, the pH gradient is reversed but a stimulation of the initial rate of Ca^{2+} transport still

Fig. 3. Relationship between the steady-state protonmotive force and the initial rate of calcium transport

Fig.a: Incubation conditions were the same as in Fig. 1. The protonmotive force was determined 10 and 20 sec after adding varying concentrations of tributyltin and the average values from duplicate measurements are given. Protonmotive force (●), membrane potential (■), pH gradient (▲).

Fig.b: Mitochondria were preincubated in the presence of varying concentrations of tributyltin in the medium described in Fig. 1 at 2°C. 25 µM $CaCl_2$ containing $^{45}Ca^{2+}$ was added and the initial rate of Ca^{2+} transport was measured as described in the Experimental section.


(a)
250
200
150
100
50
0
-50
-100
Protonmotive force (mV)
0
1
2
3
4
5
μM tributyltin
(b)
15
10
5
0
initial rate of Ca uptake
(nmoles/10 sec.mg protein)
1
2
3
4
5
μM tributyltin

occurs. This indicates that neither the magnitude nor the polarity of the pH gradient has any effect on the initial rate of Ca^{2+} transport.

Quantitative relation between the initial change in membrane potential and the initial rate of Ca^{2+} accumulation measured at different concentrations of added Ca^{2+} and in the absence and presence of tributyltin

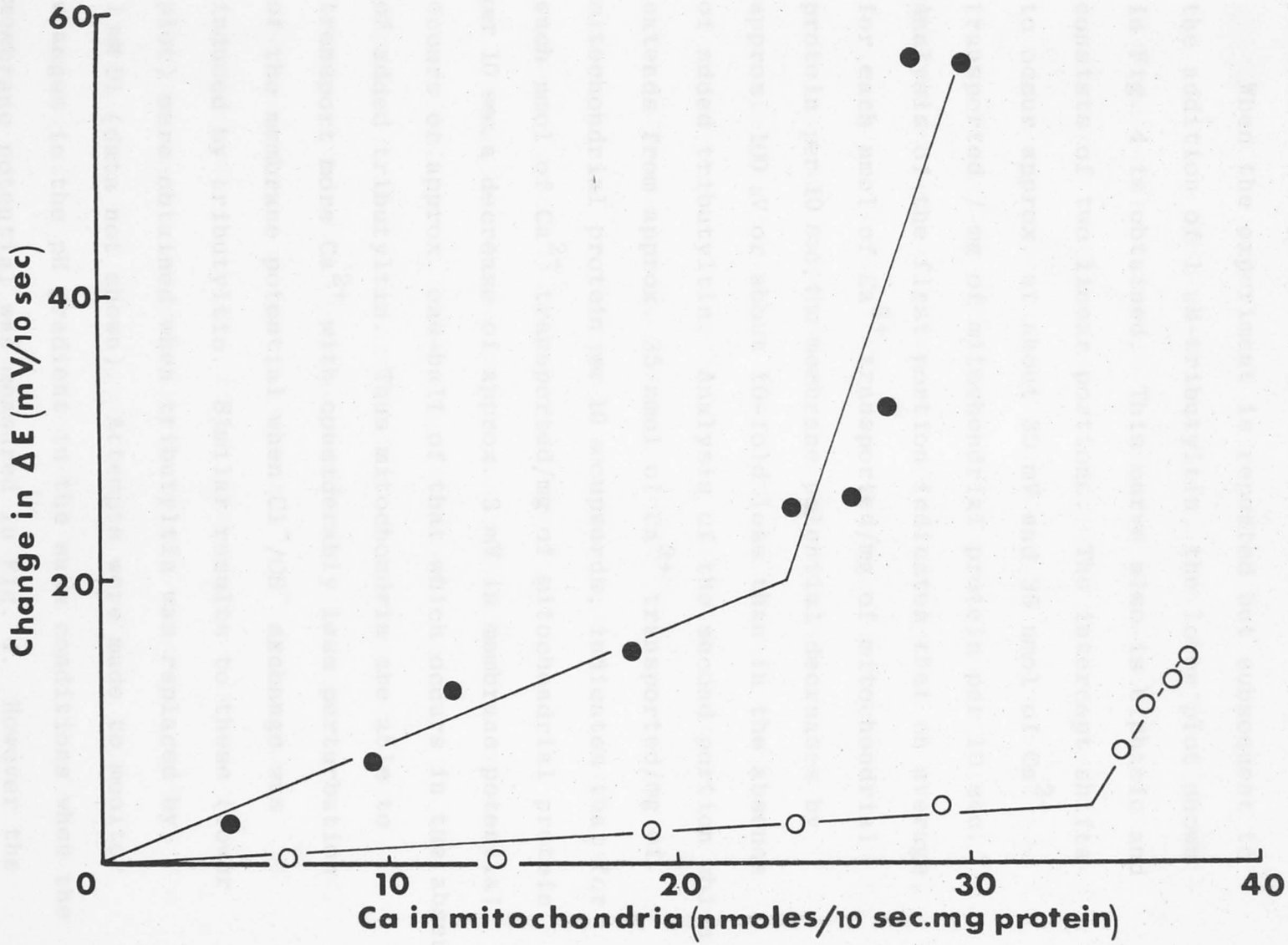
Data in Fig. 4 (upper plot) show the relation between the initial change in membrane potential and the initial rate of Ca^{2+} transport following the addition of various concentrations of Ca^{2+} to rat liver mitochondria. The relation clearly is biphasic and consists of two linear portions each exhibiting a quite different slope. The first portion indicates that concentrations of transported Ca^{2+} up to approx. 25 nmol/mg of mitochondrial protein induce a change in membrane potential that is directly proportional to the amount of Ca^{2+} transported.

A decrease in membrane potential of approx. 20 mV occurs when approx. 25 nmol of Ca^{2+}/mg of mitochondrial protein is transported in 10 sec. Generally the initial rate movement of 1nmol of Ca^{2+}/mg of mitochondrial protein per 10 sec from the EGTA-accessible to the EGTA-inaccessible space (Reed & Bygrave, 1974a; Bygrave, 1977) under the conditions, decreases the membrane potential by slightly less than 1 mV.

The second linear portion extends upwards from approx. 25 nmol of accumulated Ca^{2+}/mg of protein per 10 sec. By comparison with the first portion however, on average

Fig. 4. Relation between the initial change in membrane potential and initial rate of Ca^{2+} transport in the absence and presence of tributyltin

Incubation conditions were identical to those described in Fig. 1 except that the temperature was 9°C. Varying amounts of Ca^{2+} (10-200 µM) were added and the changes in membrane potential and Ca^{2+} transport determined by the centrifugation technique as described in the Experimental section 10 sec after initiating the reaction. Control (●); 1 µM tributyltin (○) added 30 sec before the addition of Ca^{2+}.


60
40
20
0
10
20
30
40
Change in ΔE (mV/10 sec)
Ca in mitochondria (nmoles/10 sec.mg protein)

for each nmol of Ca^{2+} accumulated, the decrease in membrane potential is approx. 6 mV.

When the experiment is repeated but subsequent to the addition of 1 μM-tributyltin, the lower plot shown in Fig. 4 is obtained. This curve also is biphasic and consists of two linear portions. The intercept shifts to occur approx. at about 30 mV and 35 nmol of Ca^{2+} transported / mg of mitochondrial protein per 10 sec. Analysis of the first portion indicates that on average, for each nmol of Ca^{2+} transported/mg of mitochondrial protein per 10 sec, the membrane potential decreases by approx. 100 μV or about 10-fold less than in the absence of added tributyltin. Analysis of the second portion which extends from approx. 35 nmol of Ca^{2+} transported/mg of mitochondrial protein per 10 secupwards, indicates that for each nmol of Ca^{2+} transported/mg of mitochondrial protein per 10 sec, a decrease of approx. 3 mV in membrane potential occurs or approx. one-half of that which occurs in the absence of added tributyltin. Thus mitochondria are able to transport more Ca^{2+} with considerably less perturbation of the membrane potential when Cl^-/OH^- exchange was induced by tributyltin. Similar results to these (lower plot) were obtained when tributyltin was replaced by 1 mM Pi (data not shown). Attempts were made to monitor changes in the pH gradient in the same conditions when the membrane potential was measured in Fig. 4. However the changes that occurred were impossible to accurately quantitate.

Effect of low concentrations of CCCP on the initial rate of Ca^{2+} transport and the initial change in membrane potential

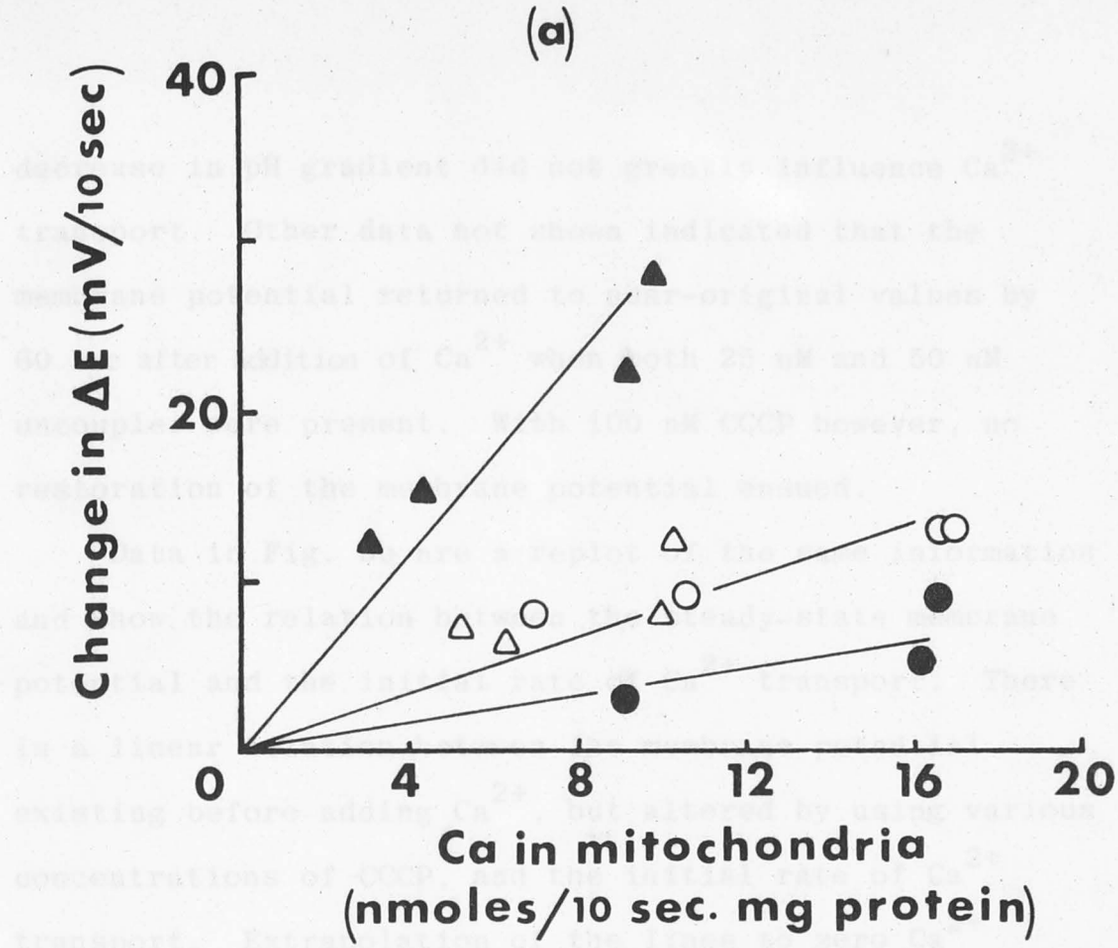
In this series of experiments the protonophore CCCP was used to perturb the protonmotive force. In preliminary experiments it was found that concentrations of the compound up to 50 nM produced a linear decrease in pH gradient from +85 mV to -50 mV; the membrane potential decreased only marginally from 155 mV to 140 mV. In the presence of 100 nM CCCP, the pH gradient and the membrane potential were -65 mV and 135 mV respectively. The experimental procedure involved preincubation of rat liver mitochondria with varying concentrations (0-100 nM) of CCCP followed by the addition of Ca^{2+} (10-40 μM). The amount of Ca^{2+} transported together with the change in membrane potential were measured.

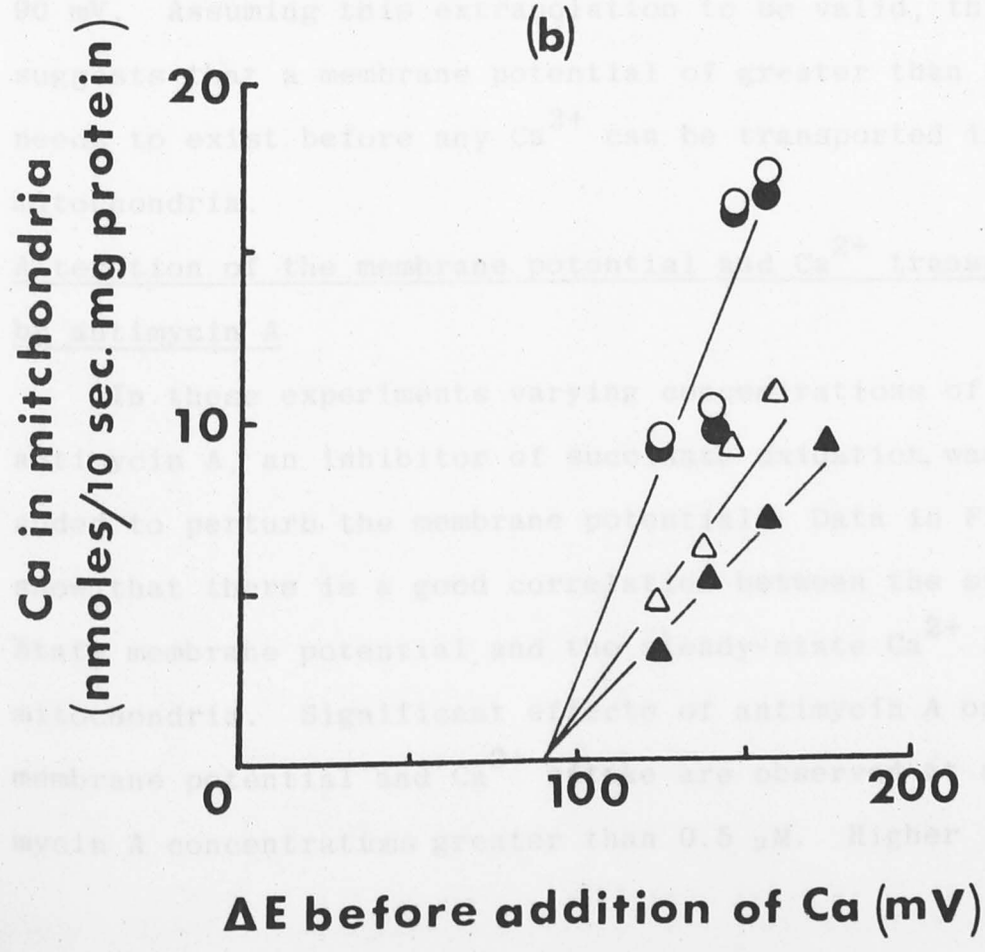
Data in Fig. 5a show results from one of three experiments each of which gave similar information. Similar to the findings in Fig. 4, there is a linear relation between the amount of Ca^{2+} transported in 10 sec and the change in membrane potential. Preincubation of mitochondria with only 25 nM CCCP leads to a relatively smaller change in membrane potential than the control for the accumulation of a given amount of Ca^{2+}. Higher concentrations of the uncoupler (50 nM and 100 nM) produce a larger change in membrane potential.

It should be noted too that almost as much Ca^{2+} is accumulated in 10 sec in the presence of low concentrations of CCCP (25 nM and 50 nM) as in its absence i.e. a

Fig. 5. Relation between the initial change in membrane potential and initial rate of Ca^{2+} transport in the absence and presence of CCCP

Incubation conditions were identical to those described in Fig. 1. Varying concentrations of Ca^{2+} (10-40 μM) were added. Control (○), 25 nM (●), 50 nM (△), and 100 nM (▲) CCCP. Fig. 5b is a replot of the data from Fig. 5a to show the relation between the steady-state membrane potential and the initial rate of Ca^{2+} transport. 10 μM (▲), 20 μM (△), 30 μM (●), and 40 μM (○) added Ca^{2+}.


(a)
40
20
0
4
8
12
16
20
Change in ΔE (mV/10 sec)
Ca in mitochondria
(nmoles/10 sec. mg protein)


(b)
20
10
0
100
200
Ca in mitchondria
(nmoles/10 sec. mg protein)
ΔE before addition of Ca (mV)

decrease in pH gradient did not greatly influence Ca^{2+} transport. Other data not shown indicated that the membrane potential returned to near-original values by 60 sec after addition of Ca^{2+} when both 25 nM and 50 nM uncoupler were present. With 100 nM CCCP however, no restoration of the membrane potential ensued.

Data in Fig. 5b are a replot of the same information and show the relation between the steady-state membrane potential and the initial rate of Ca^{2+} transport. There is a linear relation between the membrane potential existing before adding Ca^{2+}, but altered by using various concentrations of CCCP, and the initial rate of Ca^{2+} transport. Extrapolation of the lines to zero Ca^{2+} transport, intercepts the membrane potential axis at about 90 mV. Assuming this extrapolation to be valid, this suggests that a membrane potential of greater than 90 mV needs to exist before any Ca^{2+} can be transported into mitochondria.

Alteration of the membrane potential and Ca^{2+} transport by antimycin A

In these experiments varying concentrations of antimycin A, an inhibitor of succinate oxidation, was added to perturb the membrane potential. Data in Fig. 6a show that there is a good correlation between the steady-state membrane potential and the steady-state Ca^{2+} in mitochondria. Significant effects of antimycin A on the membrane potential and Ca^{2+} uptake are observed at antimycin A concentrations greater than 0.5 μM. Higher

Fig. 6. Effect of antimycin A on the membrane potential and Ca^{2+} transport in steady-state

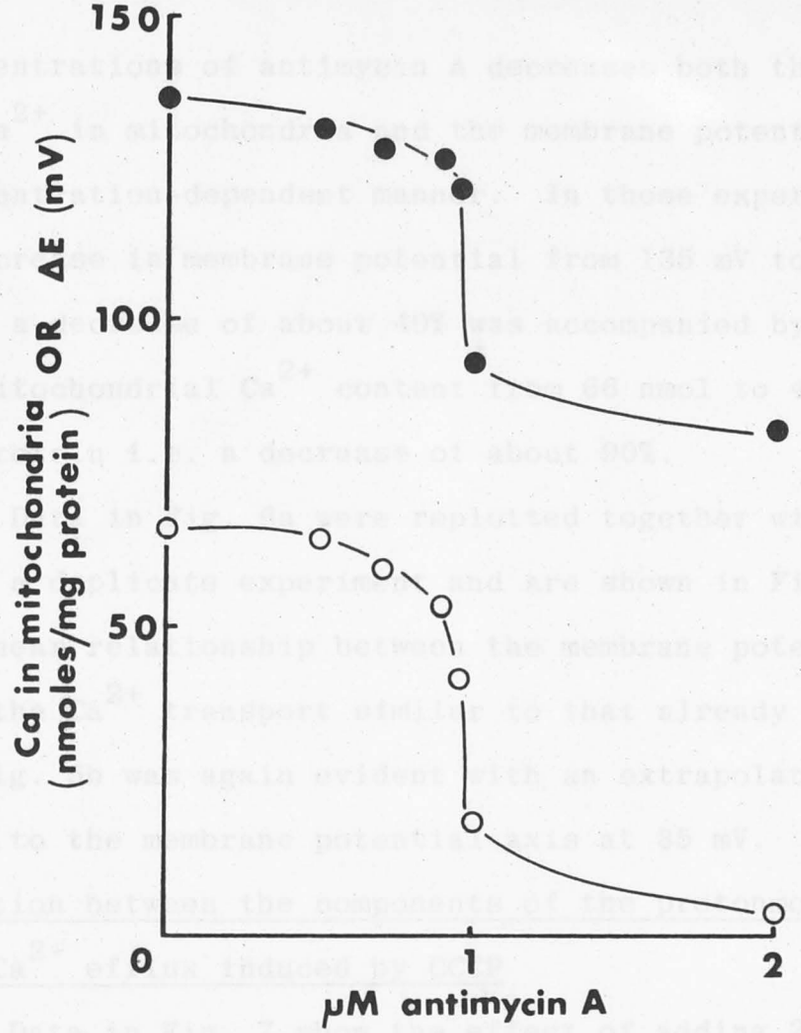
Mitochondria (1 mg/ml) was incubated in the medium described in Fig. 1 at 25°C in the presence of varying concentrations of antimycin A (0-2 μM) and 70 μM $CaCl_2$ for 7 min. Membrane potential and Ca^{2+} transport was determined as described in the Experimental Section.

Fig. a: membrane potential (●)

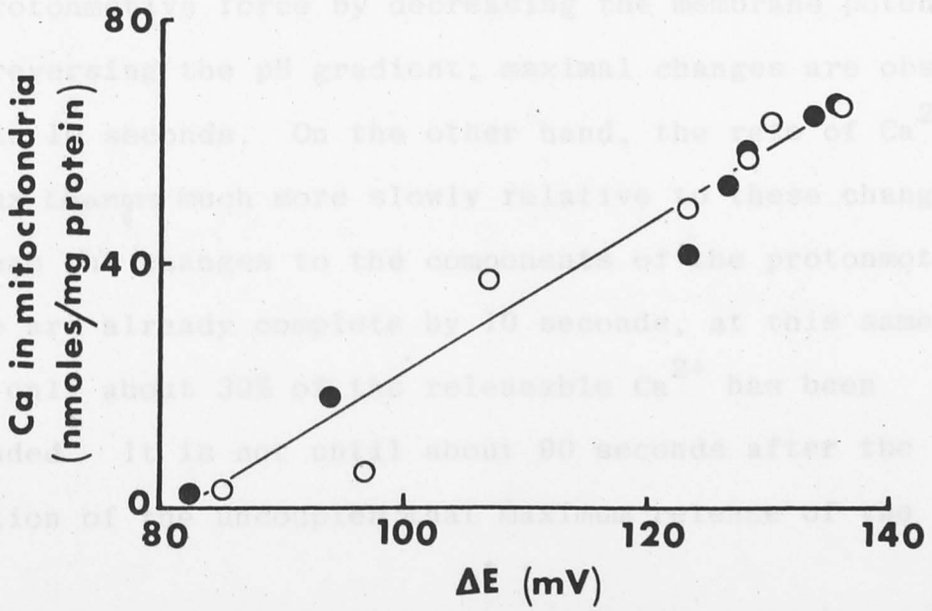
Ca^{2+} transport (○)

Fig. b is a replot of the data of Fig. a (○) and that from a duplicate experiment (●).

(a)


150
100
50
0
1
2
Ca in mitochondria OR ΔE (mV)
(nmoles/mg protein)
μM antimycin A

(b)


80
40
0
80
100
120
140
Ca in mitochondria
(nmoles/mg protein)
ΔE (mV)

concentrations of antimycin A decreases both the amount of Ca^{2+} in mitochondria and the membrane potential in a concentration-dependent manner. In these experiments a decrease in membrane potential from 135 mV to 82 mV i.e. a decrease of about 40% was accompanied by a decrease in mitochondrial Ca^{2+} content from 66 nmol to 4 nmol/mg of protein i.e. a decrease of about 90%.

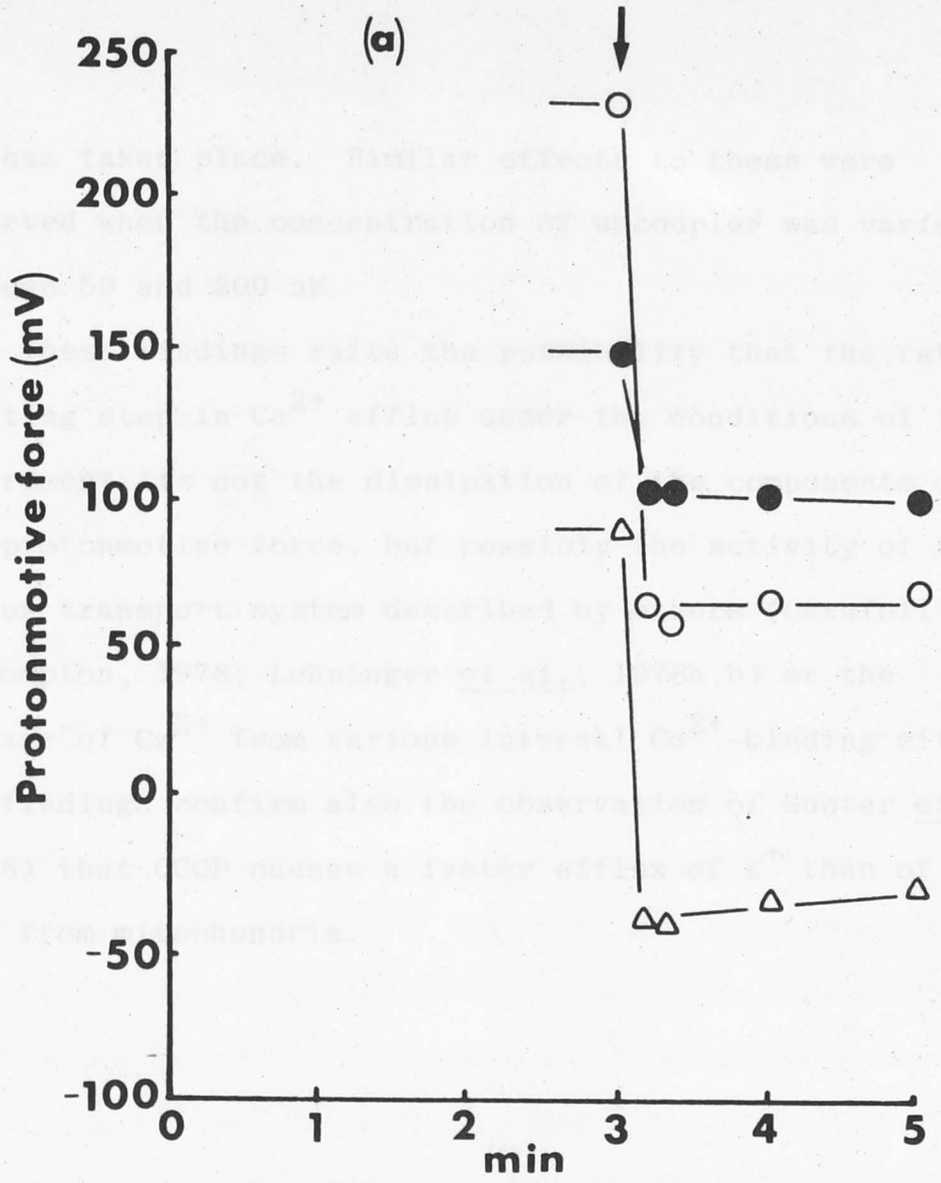
Data in Fig. 6a were replotted together with data from a duplicate experiment and are shown in Fig. 6b. A linear relationship between the membrane potential and the Ca^{2+} transport similar to that already presented in Fig. 5b was again evident with an extrapolated intercept to the membrane potential axis at 85 mV.

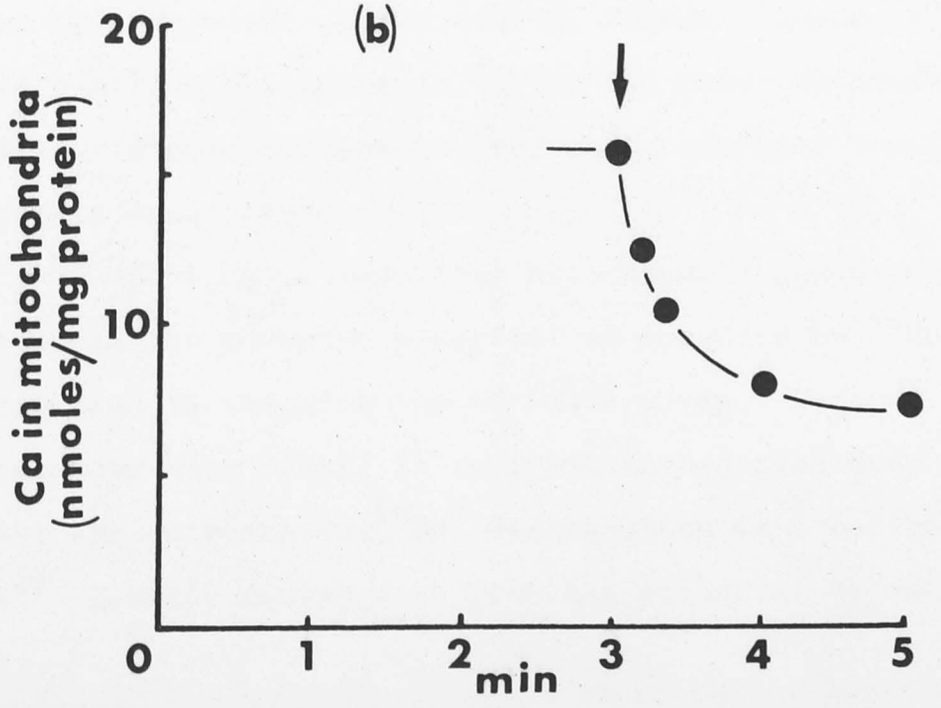
Relation between the components of the protonmotive force and Ca^{2+} efflux induced by CCCP

Data in Fig. 7 show the effect of adding 200 nM CCCP to mitochondria already loaded with Ca^{2+}, on the protonmotive force and Ca^{2+} retention by the mitochondria. Addition of the uncoupler produces a very rapid change in protonmotive force by decreasing the membrane potential and reversing the pH gradient; maximal changes are observed within 10 seconds. On the other hand, the rate of Ca^{2+} efflux changes much more slowly relative to these changes. Whereas the changes to the components of the protonmotive force are already complete by 10 seconds, at this same time only about 30% of the releasable Ca^{2+} has been extruded. It is not until about 90 seconds after the addition of the uncoupler that maximum release of the

Fig. 7. Effect of CCCP on the components of the protonmotive force and Ca^{2+} efflux

Mitochondria were preincubated for 3 min in the presence of 20 μM Ca^{2+} in the medium as in Fig. 1. CCCP (200 nM) was added (arrowed) and the components of protonmotive force (Fig. 7a) and Ca^{2+} transport (Fig. 7b) were followed as described in the Experimental section. Fig. a: membrane potential (●), pH gradient (Δ), protonmotive force (○).


(a)
250
200
150
100
50
0
-50
-100
Protonmotive force (mV)
0
1
2
3
4
5
min


(b)
20
10
Ca in mitochondria
(nmoles/mg protein)
0
1
2
3
4
5
min

ion has taken place. Similar effects to these were observed when the concentration of uncoupler was varied between 50 and 200 nM.

These findings raise the possibility that the rate-limiting step in Ca^{2+} efflux under the conditions of this experiment, is not the dissipation of the components of the protonmotive force, but possibly the activity of the efflux transport system described by others (Carafoli & Crompton, 1978; Lehninger et al., 1978a,b) or the release of Ca^{2+} from various internal Ca^{2+}-binding sites. The findings confirm also the observation of Gunter et al. (1978) that CCCP causes a faster efflux of K^{+} than of Ca^{2+} from mitochondria.

DISCUSSION

Membrane potential and Ca^{2+} transport

Implicit in the interpretation of much of the data obtained in the present work is the underlying assumption that in the presence of valinomycin, $^{86}Rb^{+}$ distribution itself across the inner mitochondrial membrane occurs in response to the membrane potential generated in the main by respiratory-chain activity. In our experiments calculated values for the membrane potential of mitochondria respiring in a buffered medium in the presence of succinate were routinely in the vicinity of 150 mV. These values are of a similar order to those obtained for instance by Nicholls (1974) also using the $^{86}Rb^{+}$ distribution technique, Mitchell & Moyle (1969a) using the K^{+} electrode technique and Åkerman (1978a) and Åkerman & Wikstrom (1976) using the safranine method. A severe limitation to the $^{86}Rb^{+}$ distribution technique and perhaps some of the others is that only those changes in the membrane potential that occur in the bulk phases are revealed. Consequently, subtle changes in charge distribution on or in either the outer or inner surface of the inner membrane may occur but will remain completely undetected.

Ca^{2+} addition to respiring mitochondria caused a decrease in the membrane potential as measured by $^{86}Rb^{+}$ distribution in the presence of valinomycin. Before interpreting this result it is important to find out whether the decrease in $^{86}Rb^{+}$ distribution is a reflection of Ca^{2+}- induced decrease in membrane potential or due to

exchange of matrix $^{86}Rb^+$ (K^+ as well) for Ca^{2+} in the presence of valinomycin (see Scarpa & Azzone, 1970). Four pieces of evidence suggest that the decrease in $^{86}Rb^+$ distribution is the result of a decrease in the membrane potential induced by Ca^{2+} rather than $Ca^{2+}/^{86}Rb^+$ exchange. Firstly during Ca^{2+}/K^+ exchange in the presence of valinomycin no movement of H^+ occurs (Azzone et al., 1977). In the present experiments an increase in pH gradient was observed (Fig. 1a). Secondly addition of Ca^{2+} to respiring mitochondria in the presence and absence of valinomycin ejected H^+ as measured with a pH electrode. In an experiment carried out under the conditions of Fig. 1 addition of 50 nmol of Ca^{2+}/mg of protein led to the ejection of 59 nmol of H^+/mg of protein in the presence of valinomycin whereas in its absence, 63.6 nmol of H^+/mg of protein was ejected. Thirdly the Cl^-/OH^- exchange mediated by tributyltin or addition of Pi caused less change in $^{86}Rb^+$ distribution for a given amount of added Ca^{2+} than in its absence (Fig. 4). Lastly Åkerman (1978a) recently reported similar Ca^{2+}-induced decreases in the membrane potential in the absence of valinomycin using the safranine technique. Hence it is concluded that the decrease in $^{86}Rb^+$ distribution induced by Ca^{2+} is due a decrease in the membrane potential.

The Ca^{2+}-induced decrease in membrane potential is due to the transport of ionic Ca^{2+} and provides direct evidence for the electrophoretic nature of the ion transport by the organelle. This experiment however does

not show the net charge transfer during Ca^{2+} transport. Experiments shown in the previous chapter (see P 64) indicate that the transport of Ca^{2+} was equivalent to the transfer of two positive charges. Hence the movement of Ca^{2+} carrying two positive charges into the mitochondrial matrix occurs in response to the membrane potential existing across the inner membrane. The precise mechanism of coupling between the membrane potential and Ca^{2+} transport remains largely unknown.

Data in a number of experiments reported in this chapter indicated that there was a qualitative correlation between the altered membrane potential induced by tributyltin (Fig. 3), CCCP (Fig. 5), antimycin A (Fig. 6) and Ca^{2+} transport. However the relationship between the changes in the membrane potential and Ca^{2+} transport, was non-linear. In Fig. 2 it was seen that following the addition of each concentration of tributyltin, the extra Ca^{2+} transport was completed considerably more quickly than the re-equilibration of membrane potential. Fig. 3 revealed that although prior addition of 1 μM-tributyltin increased membrane potential by only some 10%, the initial rate of Ca^{2+} transport was stimulated approx. 60%. Fig. 4 showed that the correlation between the initial change in the membrane potential and the corresponding change in Ca^{2+} transport was biphasic both in the presence and absence of tributyltin. Alterations in the steady-state membrane potential and Ca^{2+} transport induced by antimycin A (Fig. 6a) showed that a 40% decrease in the

membrane potential was associated with a 90% decrease in the Ca^{2+} transport.

These results along with that in Fig. 7 which indicates that the release Ca^{2+} from mitochondria is slower than the loss of membrane potential, support the idea that the driving force (membrane potential) may not limit Ca^{2+} movements (Bygrave et al., 1978a see also Chapter 6).

pH gradient and mitochondrial Ca^{2+} transport

The present work shows that in addition to a decrease in the membrane potential there is an increase in the pH gradient, following the addition of Ca^{2+} to respiring mitochondria (Fig. 1a). Acetate distribution was used to calculate the pH gradient. The possibility of accumulation of acetate with Ca^{2+} similar to that occurring with β-hydroxybutyrate described by Brand et al. (1976a), accounting for the enhanced acetate accumulation, hence the pH gradient, appears unlikely because of the following reasons. Firstly the distribution of acetate returned back to its original value with time after a pulse of Ca^{2+} (Fig. 1a) though Ca^{2+} was retained by the mitochondria (Fig. 1b). Secondly a decrease in acetate distribution resulting from the Cl^-/OH^- exchange induced by tributyltin was observed (Fig. 2a) even though an increased amount of Ca^{2+} was transported and retained by the organelle (Fig. 2b). Lastly Mitchell & Moyle (1969a) observed similar Ca^{2+}-induced increase in pH gradient measured with a pH electrode.

Data from experiments in the present study provide

some indication that the magnitude of pH gradient has little influence on the initial rate of Ca^{2+} transport. Thus altering the pH gradient from 85 mV negative inside to about 20 mV positive inside by the addition of low concentrations of tributyltin enhanced the initial rate of Ca^{2+} transport (Fig. 3) whilst a similar alteration in the pH gradient with CCCP resulted in a slightly lowered initial rate of Ca^{2+} transport (Fig. 5).

On the other hand, the data revealed that in the presence of tributyltin or Pi (Fig. 4) or CCCP (Fig. 5) the membrane potential was perturbed to a much smaller extent in the course of movement of a given amount of Ca^{2+} into mitochondria. This raises the question as to whether the pH gradient normally existing across the inner mitochondrial membrane infact may actually restrain at least to some degree the initial rate of uptake of Ca^{2+} by rat liver mitochondria.

Much of the data presented in this chapter have recently been confirmed by Åkerman (1978a) and Nicholls (1978a,b). These workers demonstrated similar changes in membrane potential and pH gradient on adding Ca^{2+} to rat liver, rat heart and guinea pig cerebral cortex mitochondria.

CHAPTER 6

THE INFLUENCE OF THE PROTON CONCENTRATION ON CALCIUM-ION FLUXES ACROSS THE INNER MITOCHONDRIAL MEMBRANE OF RAT LIVER

THE INFLUENCE OF THE PROTON CONCENTRATION ON CALCIUM-ION FLUXES ACROSS THE INNER MITOCHONDRIAL MEMBRANE OF RAT LIVER

INTRODUCTION

Because Ca^{2+} is considered to play an important role in the regulation of cellular metabolism (Bygrave, 1967; Rasmussen, 1970; Carafoli, 1974), much interest currently centres on the various ways in which the intracellular concentration of the ion is itself regulated. There is now a strong belief that mitochondria in many tissues and species are important in such regulation (for reviews see Lehninger et al., 1967; Mela, 1977; Bygrave, 1977, 1978a; Carafoli & Crompton, 1978) and as a consequence important in controlling metabolic activities sensitive to Ca^{2+} (see e.g. Meli & Bygrave, 1972).

Movement of Ca^{2+} across the inner mitochondrial membrane is bidirectional in the sense that separate pathways are considered to be responsible for the inward and outward translocation of the ion (see e.g. Crompton & Heid, 1978; Nicholls, 1978a,b; Carafoli, 1979 and the many references quoted in these reports). These pathways in effect contribute to a Ca^{2+}-translocation cycle that permits considerable flexibility, sensitivity and amplification in the control of mitochondrial Ca^{2+} fluxes (Bygrave, 1978a,b; Crompton & Heid, 1978; Nicholls, 1978b) not unlike that ascribed to the role of substrate-cycling in metabolic regulation (Newsholme & Crabtree, 1976).

Recent reports that glucagon (Hughes & Barritt, 1978;

Prpic et al., 1978) and α-adrenergic agonists (Taylor et al., 1980) are capable of inducing significant changes in both Ca^{2+} influx and efflux within minutes of their administration to perfused rat livers so as to strongly promote mitochondrial Ca^{2+} retention, adds strength to the argument that the mitochondrial Ca^{2+}-translocation cycle plays an important role in the regulation of cell metabolism (see e.g. Bygrave, 1978a,b).

Besides providing new information about the targets of action of specific hormones in liver cells these findings in turn can be exploited to provide further information about aspects of the basic mechanism underlying the physiological control of this cycle. In this regard it seems potentially pertinent that the action in vivo of glucagon (Titheradge & Coore, 1976; Halestrap, 1978; Prpic et al., 1978) and of α-adrenergic agonists (Taylor et al., 1980), on liver mitochondria leads to an increase in the transmembrane pH gradient, concomitant with the induced increase in mitochondrial Ca^{2+} retention. We regarded this point to be worthy of further study.

Initially we considered the question of how the transmembrane pH gradient might influence Ca^{2+} fluxes across the inner membrane of mitochondria isolated from liver of control rats. It soon became apparent that such an in-depth systematic study has not been described. Previous reports have shown that the efflux of Ca^{2+} from pre-loaded mitochondria as measured by H^{+} uptake, and induced by inhibiting the generation of energy for ion

transport, is sensitive to variations in the pH of the medium (Rossi et al., 1966b); that the initial rate of Ca^{2+} transport is sensitive to changes in the pH of the medium (Scarpa & Azzone, 1970; Reed & Bygrave, 1975b); that both the apparent Ca^{2+} conductance of the inner mitochondrial membrane and the ability of mitochondria to maintain extramitochondrial free Ca^{2+} in the steady-state are susceptible to changes in the pH of the medium (Heaton & Nicholls, 1976; Nicholls, 1978b) and that mitochondria release Ca^{2+} transiently when the pH of the incubation medium is decreased by adding a pulse of HCl (Åkerman, 1978c).

In this work we examined therefore the influence of varying the pH of the incubation medium on a range of energy-linked reactions in rat liver mitochondria that involve the movement of Ca^{2+} across the inner membrane.

RESULTS

Effect of medium pH on Ca^{2+}-induced stimulation of succinate oxidation by rat liver mitochondria

Data in Fig. 1 show the effect of medium pH on the Ca^{2+}-induced stimulation of succinate oxidation by rat liver mitochondria. Immediately following the addition of Ca^{2+} to mitochondria an increase in respiration occurs reflecting accumulation of the ion (Rossi & Lehninger, 1964). However the point at which the stimulated rate of respiration ceases depends on the pH of the medium. With a medium pH of 6.0 the stimulated rate of respiration shows no sign of abating to the rate existing before Ca^{2+} addition, whereas at pH 7.4 the rate of respiration returns to the original resting rate for approx. 2 min. It then increases to approach near-maximal rates after about 2 to 4 min. The time for which the rate of respiration increases to maximal (uncoupled) levels is a function of the medium pH (data not shown). When the pH of the medium is 8.0, respiration returns to the 'resting' rate immediately following the Ca^{2+}-induced respiratory 'jump' and remains so until anaerobiosis is reached (approx. 30 min).

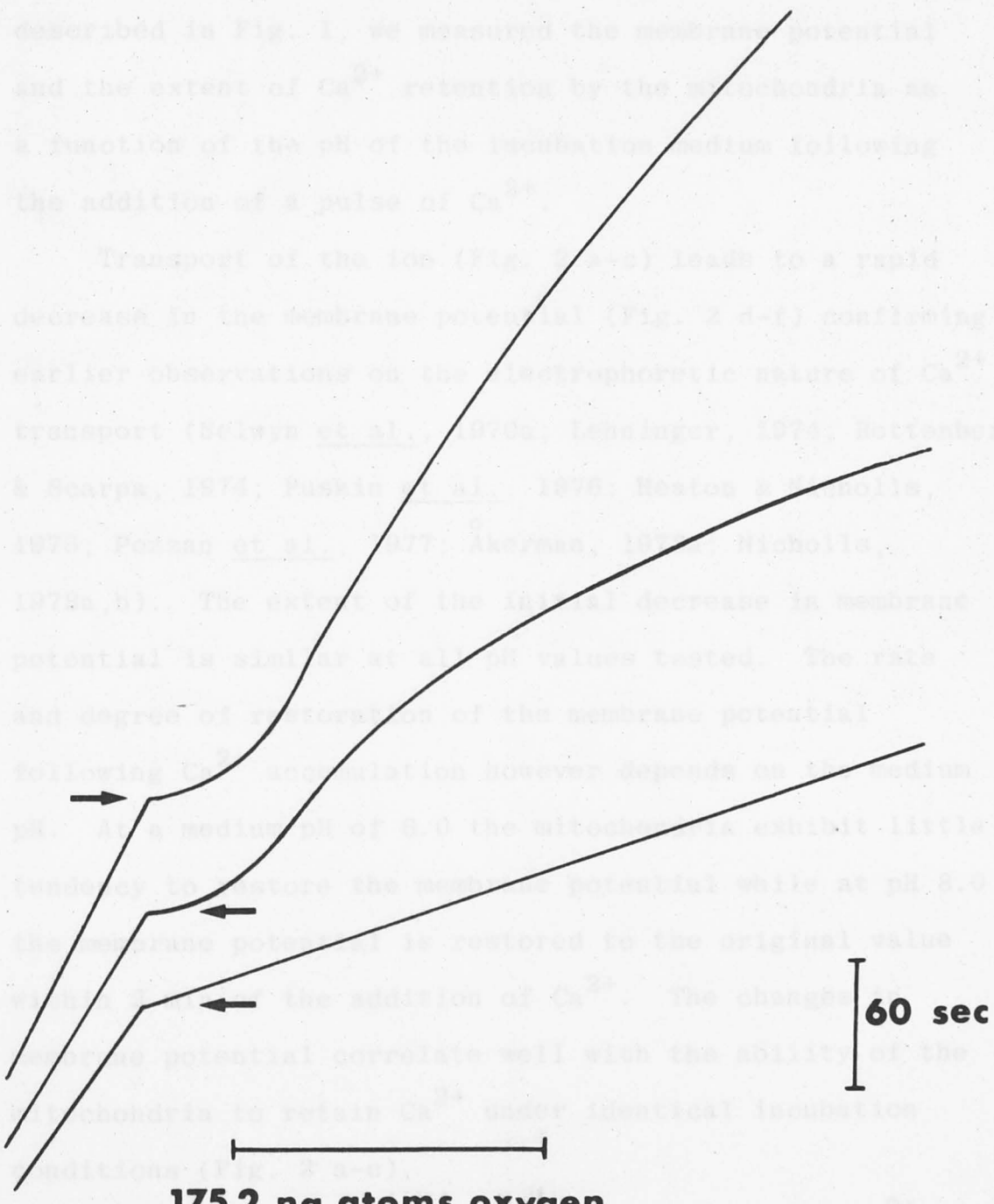
The differing sensitivites of Ca^{2+}-induced uncoupling to the medium pH were then assessed by the direct measurement of Ca^{2+} accumulation by rat liver mitochondria.

Effect of medium pH on Ca^{2+}-induced changes in the membrane potential under conditions of Ca^{2+}-uncoupling

The energy required for Ca^{2+} transport into mitochondria is provided by the membrane potential, negative inside,

Fig. 1. Effect of pH on Ca^{2+}-induced uncoupling

Oxygen uptake was measured polarographically as described in the Experimental Section. The reaction medium contained in a total volume of 2.0 ml, 230 mM-sucrose, 5 mM-Hepes, 0.5 mM-KCl, 5 mM-sodium succinate 2 mM-Pi and 5 μM-rotenone. Mitochondria were present at a concentration of 1 mg/ml and the temperature was 25°C. 100 μM $CaCl_2$ was added at the point indicated by the arrow. The pH of the solution was adjusted before adding mitochondria using NaOH and was 6.0 (lower curve), 7.4 (middle curve) or 8.0 (upper curve). 1 ml of the medium was assumed to contain 444 ng atoms of oxygen.


60 sec
175.2 ng atoms oxygen

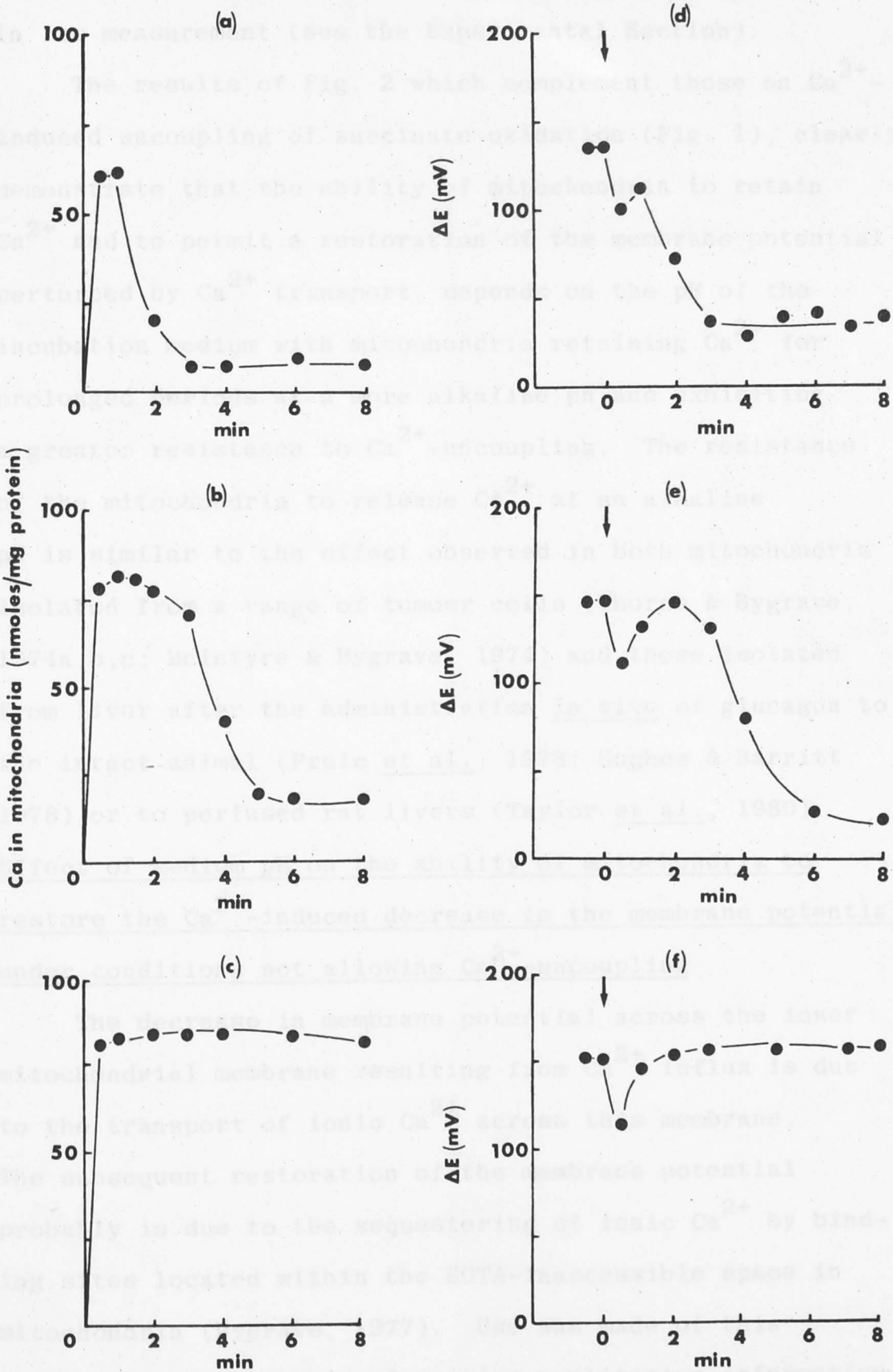
that exists across the inner mitochondrial membrane (Mitchell & Moyle, 1969a). Using conditions similar to those described in Fig. 1, we measured the membrane potential and the extent of Ca^{2+} retention by the mitochondria as a function of the pH of the incubation medium following the addition of a pulse of Ca^{2+}.

Transport of the ion (Fig. 2 a-c) leads to a rapid decrease in the membrane potential (Fig. 2 d-f) confirming earlier observations on the electrophoretic nature of Ca^{2+} transport (Selwyn et al., 1970a; Lehninger, 1974; Rottenberg & Scarpa, 1974; Puskin et al., 1976; Heaton & Nicholls, 1976; Pozzan et al., 1977; Åkerman, 1978a; Nicholls, 1978a,b). The extent of the initial decrease in membrane potential is similar at all pH values tested. The rate and degree of restoration of the membrane potential following Ca^{2+} accumulation however depends on the medium pH. At a medium pH of 6.0 the mitochondria exhibit little tendency to restore the membrane potential while at pH 8.0 the membrane potential is restored to the original value within 2 min of the addition of Ca^{2+}. The changes in membrane potential correlate well with the ability of the mitochondria to retain Ca^{2+} under identical incubation conditions (Fig. 2 a-c).

It should be pointed out that the release of Ca^{2+} is accompanied by large amplitude swelling of the mitochondria (see later) and hence the membrane potential measured after the release of Ca^{2+} at pH 6.0 and 7.1 may be overestimated since a fixed matrix volume was assumed

Fig. 2. Ca^{2+}-induced uncoupling and loss of the membrane potential

Mitochondria were incubated in 4.0 ml of medium as described in Fig. 1 for 2 min at 25°C in the presence of 10 µM $^{86}RbCl$ (0.12 µCi/ml), [U-^{14}C] sucrose (0.4 µCi/ml) and 0.5 µM valinomycin. Samples were removed for determination of membrane potential. At time zero in the figure 100 µM-$CaCl_2$ was added and at appropriate intervals samples were removed. The membrane potential was calculated as described in the Experimental Section. Ca^{2+} transport was followed under identical conditions, in the absence of the above radiochemicals, by using $^{45}Ca^{2+}$ as described in the Experimental Section. The pH of the medium before adding Ca^{2+} was 6.0 (a & d), 7.1 (b & e) or 8.0 (c & f). ΔE= membrane potential.


(a)
(b)
(c)
(d)
(e)
(f)
Ca in mitochondria (nmoles/mg protein)
ΔE (mV)
min
100
50
0
200
2
4
6
8

in its measurement (see the Experimental Section).

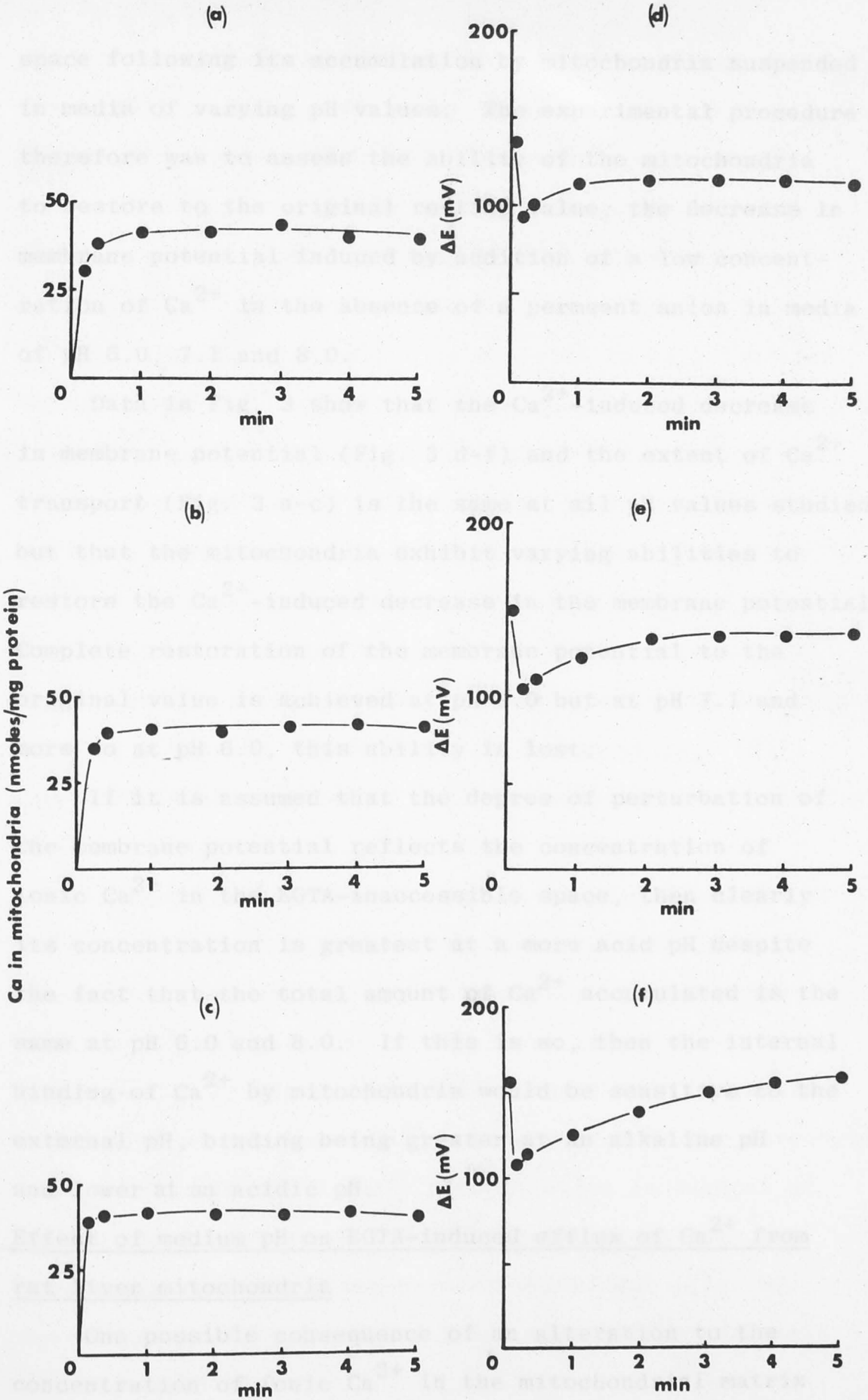
The results of Fig. 2 which complement those on Ca^{2+}-induced uncoupling of succinate oxidation (Fig. 1), clearly demonstrate that the ability of mitochondria to retain Ca^{2+} and to permit a restoration of the membrane potential perturbed by Ca^{2+} transport, depends on the pH of the incubation medium with mitochondria retaining Ca^{2+} for prolonged periods at a more alkaline pH and exhibiting a greater resistance to Ca^{2+}-uncoupling. The resistance of the mitochondria to release Ca^{2+} at an alkaline pH is similar to the effect observed in both mitochondria isolated from a range of tumour cells (Thorne & Bygrave, 1974a,b,c; McIntyre & Bygrave, 1974) and those isolated from liver after the administration *in vivo* of glucagon to the intact animal (Prpic *et al.*, 1978; Hughes & Barritt, 1978) or to perfused rat livers (Taylor *et al.*, 1980).

Effect of medium pH on the ability of mitochondria to restore the Ca^{2+}-induced decrease in the membrane potential under conditions not allowing Ca^{2+}-uncoupling

The decrease in membrane potential across the inner mitochondrial membrane resulting from Ca^{2+} influx is due to the transport of ionic Ca^{2+} across this membrane. The subsequent restoration of the membrane potential probably is due to the sequestering of ionic Ca^{2+} by binding sites located within the EGTA-inaccessible space in mitochondria (Bygrave, 1977). Use was made of this observation in the hope of gaining qualitative information about the status of ionic Ca^{2+} in the EGTA-inaccessible

Fig. 3. Effect of pH on the Ca^{2+}-induced decrease in the membrane potential

Mitochondria were equilibrated aerobically at 25°C for 2 min in the absence of Pi in the medium described in Fig. 2. 50 μM $CaCl_2$ was added at time zero and at appropriate time intervals samples were removed for membrane potential, the determination of which is described in the Experimental Section. Ca^{2+} transport was followed under identical conditions as described in the Experimental Section. The pH of the medium was 6.0 (a & d), 7.1 (b & e) or 8.0 (c & f). ΔE= membrane potential.


(a)
(b)
(c)
(d)
(e)
(f)
Ca in mitochondria (nmoles/mg protein)
ΔE (mV)
min
50
25
200
100
0
1
2
3
4
5

space following its accumulation by mitochondria suspended in media of varying pH values. The experimental procedure therefore was to assess the ability of the mitochondria to restore to the original resting value, the decrease in membrane potential induced by addition of a low concentration of Ca^{2+} in the absence of a permeant anion in media of pH 6.0, 7.1 and 8.0.

Data in Fig. 3 show that the Ca^{2+}-induced decrease in membrane potential (Fig. 3 d-f) and the extent of Ca^{2+} transport (Fig. 3 a-c) is the same at all pH values studied but that the mitochondria exhibit varying abilities to restore the Ca^{2+}-induced decrease in the membrane potential. Complete restoration of the membrane potential to the original value is achieved at pH 8.0 but at pH 7.1 and more so at pH 6.0, this ability is lost.

If it is assumed that the degree of perturbation of the membrane potential reflects the concentration of ionic Ca^{2+} in the EGTA-inaccessible space, then clearly its concentration is greatest at a more acid pH despite the fact that the total amount of Ca^{2+} accumulated is the same at pH 6.0 and 8.0. If this is so, then the internal binding of Ca^{2+} by mitochondria would be sensitive to the external pH, binding being greater at an alkaline pH and lower at an acidic pH.

Effect of medium pH on EGTA-induced efflux of Ca^{2+} from rat liver mitochondria

One possible consequence of an alteration to the concentration of ionic Ca^{2+} in the mitochondrial matrix

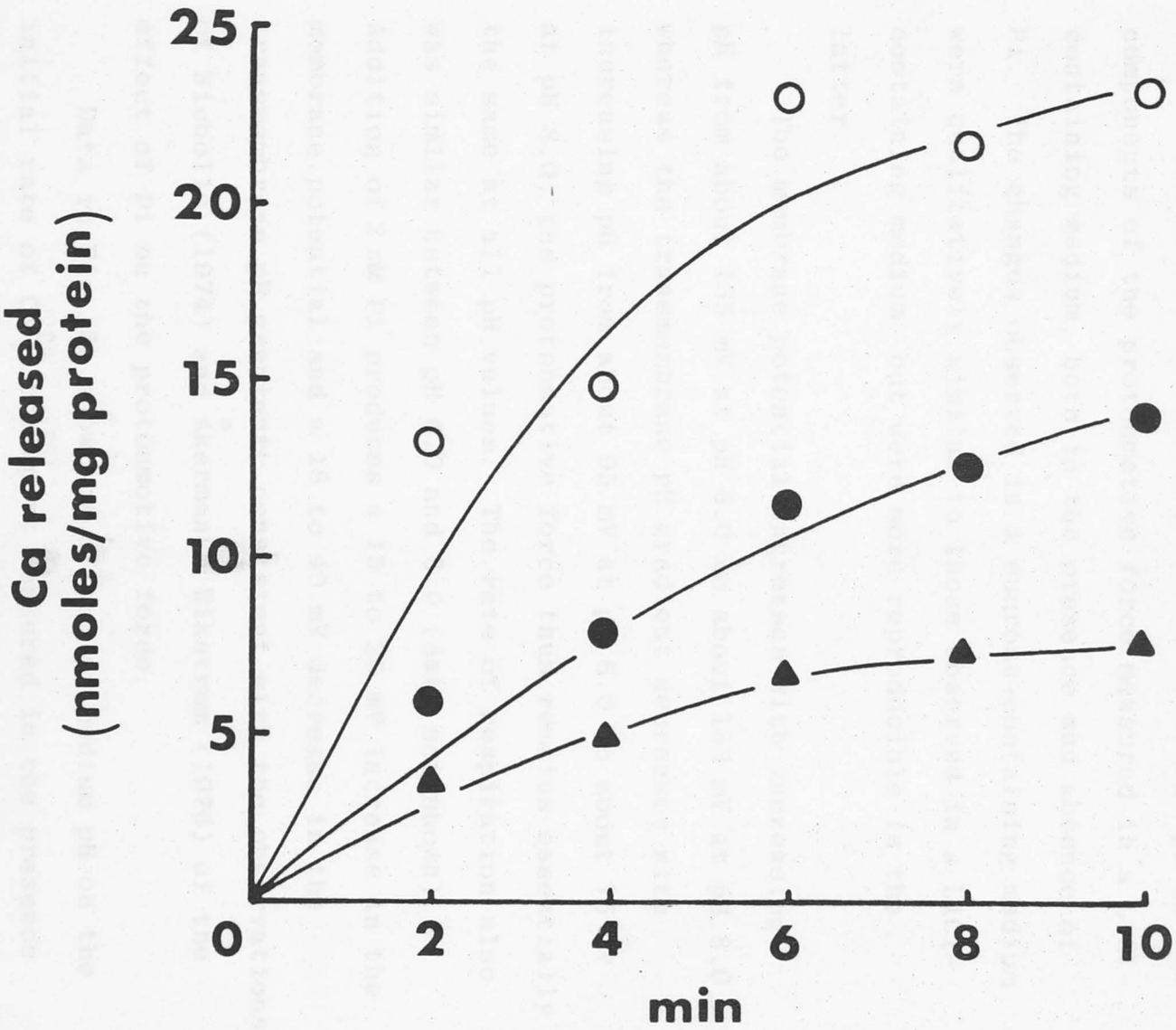
will be an alteration to the rate of Ca^{2+}-cycling, which presumably is sensitive, among other factors, to the internal and external free Ca^{2+} concentration. The external ionic Ca^{2+} can be readily reduced to very low concentrations by the addition of EGTA and hence the efflux of Ca^{2+} induced by this substance (Reed & Bygrave, 1974b; Bygrave, 1977) might be expected to be a function of the ionic Ca^{2+} concentration in the mitochondrial matrix.

Data in Fig. 4 show the effect of medium pH on the EGTA-induced efflux of Ca^{2+} from rat liver mitochondria. In these experiments mitochondria were pre-loaded with Ca^{2+} at various pH values, and then EGTA-Tris (pH adjusted to that of the medium) was added to lower the ionic Ca^{2+} concentration in the medium. In confirmation of the studies of Reed & Bygrave (1974b) and of Pozzan et al. (1977) the EGTA-induced efflux of Ca^{2+} was sensitive to Ruthenium Red (see Chapter 7). It is clear that the rate of EGTA-induced Ca^{2+} efflux is highest at pH 6.0 and lowest at pH 8.0. The rate of efflux was more rapid at higher incubation temperatures and the pattern of efflux was similar to that observed at the lower temperatures (data not shown). If the rate of EGTA-induced Ca^{2+} efflux is a function of the ionic Ca^{2+} concentration in the mitochondrial matrix, then the ionic concentration is highest at pH 6.0 and lowest at pH 8.0, fully consistent with the conclusion reached in the earlier experiment (Fig. 3). However as will be discussed below alternative arguments can be raised also.

Fig. 4. EGTA-induced efflux of Ca^{2+}

100 μM $CaCl_2$ containing $^{45}CaCl_2$ (0.5 μCi) was added to mitochondria incubated for 1 min at 3-4°C in the medium described in Fig. 1 in the absence of Pi. 10 min after adding Ca^{2+}, 2 mM-EGTA (pH adjusted to that of the medium with tris) was added at time zero in the Fig. Samples were removed at appropriate time intervals for measurement of Ca^{2+} transport as described in the Experimental Section.

The pH of the medium before adding mitochondria was 6.0 (○), 7.1 (●), or 8.0 (▲).


Ca released
(nmoles/mg protein)
25
20
15
10
5
0
2
4
6
8
10
min

Effect of pH on the components of the protonmotive force and on the initial rate of Ca^{2+} transport in the presence and absence of Pi

Data in Fig. 5a show the effect of medium pH on the components of the protonmotive force measured in a LiCl-containing medium, both in the presence and absence of Pi. The changes observed in a sucrose-containing medium were qualitatively similar to those observed in a LiCl-containing medium, but were more reproducible in the latter.

The membrane potential increases with increasing pH from about 135 mV at pH 6.0 to about 153 mV at pH 8.0, whereas the transmembrane pH gradient decreases with increasing pH from about 95 mV at pH 6.0 to about 75 mV at pH 8.0; the protonmotive force thus remains essentially the same at all pH values. The rate of respiration also was similar between pH 6.0 and 8.0 (data not shown). Addition of 2 mM Pi produces a 15 to 25 mV increase in the membrane potential and a 15 to 40 mV decrease in the transmembrane pH gradient, consistent with the observations of Nicholls (1974) and Åkerman & Wikstrom (1976) of the effect of Pi on the protonmotive force.

Data in Fig. 5b show the effect of medium pH on the initial rate of Ca^{2+} transport measured in the presence and absence of Pi. The initial rate of Ca^{2+} transport increases with increasing pH from 6.0 until it reaches a maximum near 7.5 both in the absence and presence of Pi. The anion stimulates the initial rate of Ca^{2+} transport

Fig. 5. Effect of pH on the components of the protonmotive force and on the initial rate of Ca^{2+} transport

Fig. a: Mitochondria (1 mg/ml) were incubated aerobically at 25°C for 2 min in a medium containing 150 mM-LiCl, 0.5 mM-KCl, 5 mM-sodium succinate, 5 µM-rotenone, 0.5 µM-valinomycin, 10 µM-^{86}RbCl (0.12 µCi/ml), 50 µM-[^{14}C]-methylamine (0.3 µCi/ml) and 50 µM-sodium [^{3}H]-acetate (1.2 µCi/ml). Samples were removed for determination of protonmotive force. 2 mM-Pi was then added, and 1 min later samples were removed for protonmotive force determination as described in the Experimental Section.

protonmotive force (▲)

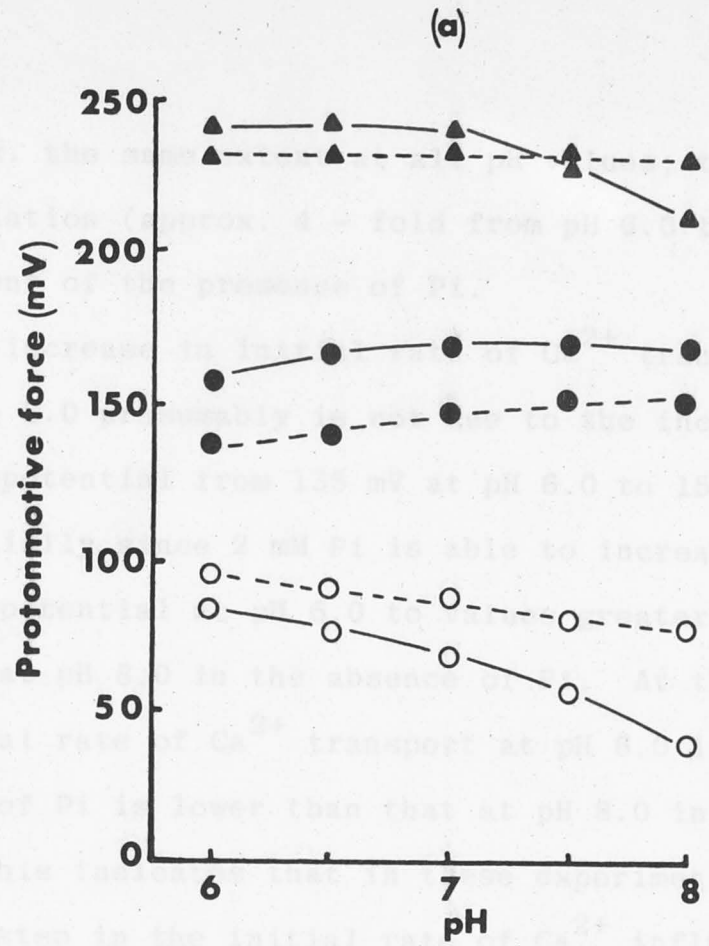
membrane potential (●)

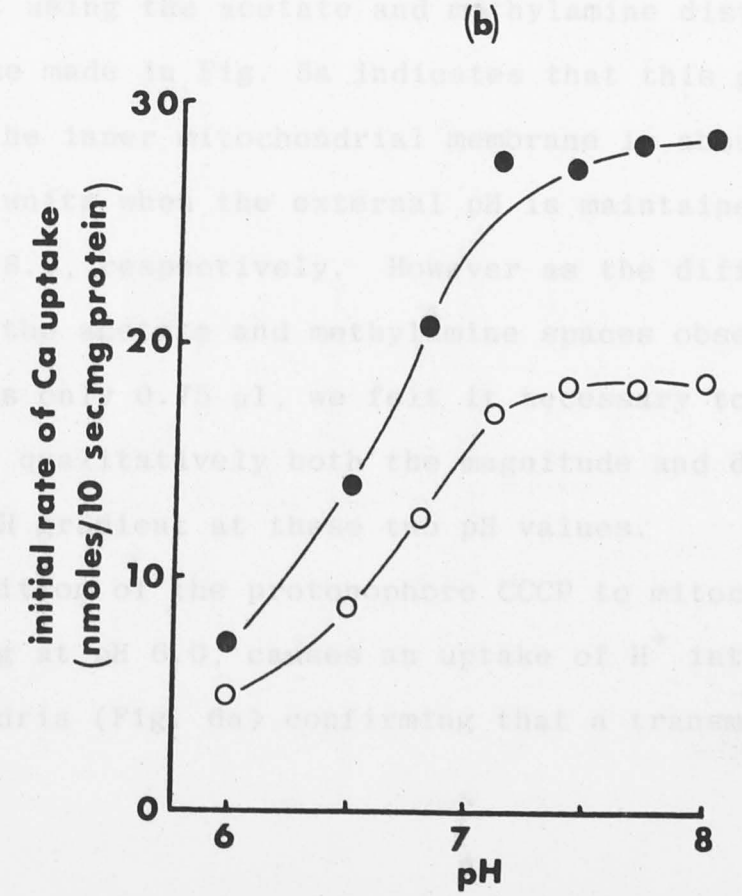
pH gradient (○)

Pi absent, discontinuous lines

Pi present, continuous lines

Fig. b: Mitochondria (1 mg/ml) were incubated at 4°C in the medium described above (Fig. 5a) in the presence (●) and absence (○) of 2 mM-Pi. 100 µM-$^{45}CaCl_2$ (0.3 µCi/ml) was added and Ca^{2+} transport was measured as described in the Experimental Section.


(a)
250
200
150
100
50
0
Protonmotive force (mV)
6
7
8
pH


(b)
30
20
10
0
initial rate of Ca uptake
(nmoles/10 sec.mg protein)
6
7
8
pH

to approx. the same extent at all pH values; the extent of stimulation (approx. 4 - fold from pH 6.0 to 7.5) is independent of the presence of Pi.

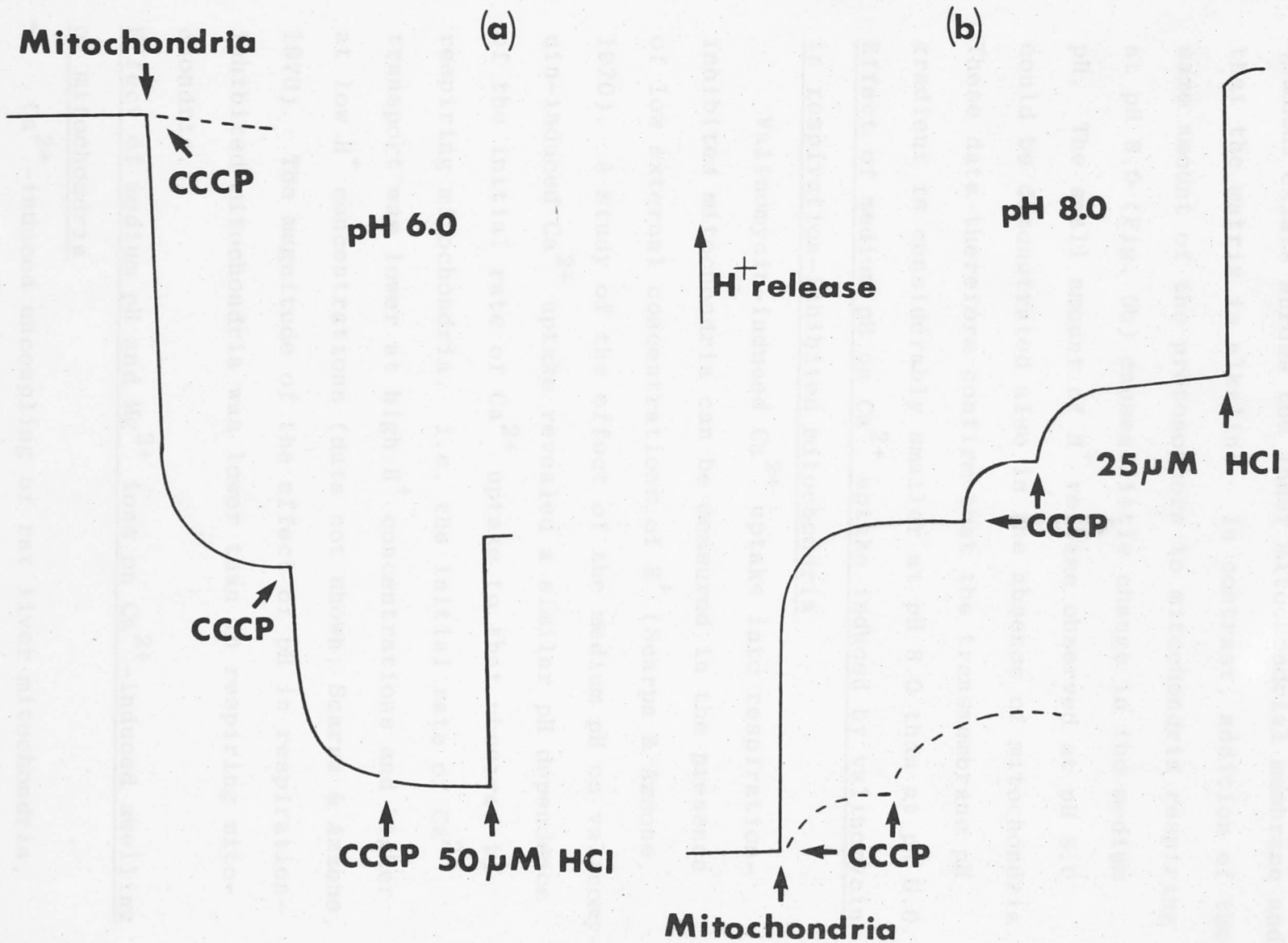
The increase in initial rate of Ca^{2+} transport from pH 6.0 to 8.0 presumably is not due to the increase in membrane potential from 135 mV at pH 6.0 to 153 mV at pH 8.0 especially since 2 mM Pi is able to increase the membrane potential at pH 6.0 to values greater than that observed at pH 8.0 in the absence of Pi. At the same time the initial rate of Ca^{2+} transport at pH 6.0 in the presence of Pi is lower than that at pH 8.0 in the absence of Pi. This indicates that in these experiments the rate-limiting step in the initial rate of Ca^{2+} influx most likely is not the driving force i.e. the membrane potential.

The direct measurement of the transmembrane pH gradient using the acetate and methylamine distribution technique made in Fig. 5a indicates that this gradient across the inner mitochondrial membrane is about 1.4 and 0.68 pH units when the external pH is maintained at pH 6.0 and 8.0, respectively. However as the difference between the acetate and methylamine spaces observed at pH 8.0 is only 0.75 µl, we felt it necessary to confirm at least qualitatively both the magnitude and direction of the pH gradient at these two pH values.

Addition of the protonophore CCCP to mitochondria respiring at pH 6.0, causes an uptake of H^+ into the mitochondria (Fig. 6a) confirming that a transmembrane pH

Fig. 6. Uncoupler-induced proton movements

The incubation medium was exactly as described in Fig. 1. Mitochondria (1 mg/ml) were added at the point indicated by the arrow and after the recorder pen had stabilized CCCP (2 μM) was added as indicated. Standard HCl was used to calibrate the pH electrode as shown in the Fig. The temperature was 25°C. Discontinuous line, mitochondria absent.


(a)
Mitochondria
CCCP
pH 6.0
CCCP
CCCP
50 μM HCl
(b)
pH 8.0
H+ release
25 μM HCl
CCCP
CCCP
Mitochondria

gradient exists across the inner mitochondrial membrane and that the matrix is alkaline. In contrast, addition of the same amount of the protonophore to mitochondria respiring at pH 8.0 (Fig. 6b) causes little change in the medium pH. The small amount of H^+ release observed at pH 8.0 could be demonstrated also in the absence of mitochondria. These data therefore confirm that the transmembrane pH gradient is considerably smaller at pH 8.0 than at pH 6.0.

Effect of medium pH on Ca^{2+} uptake induced by valinomycin in respiration-inhibited mitochondria

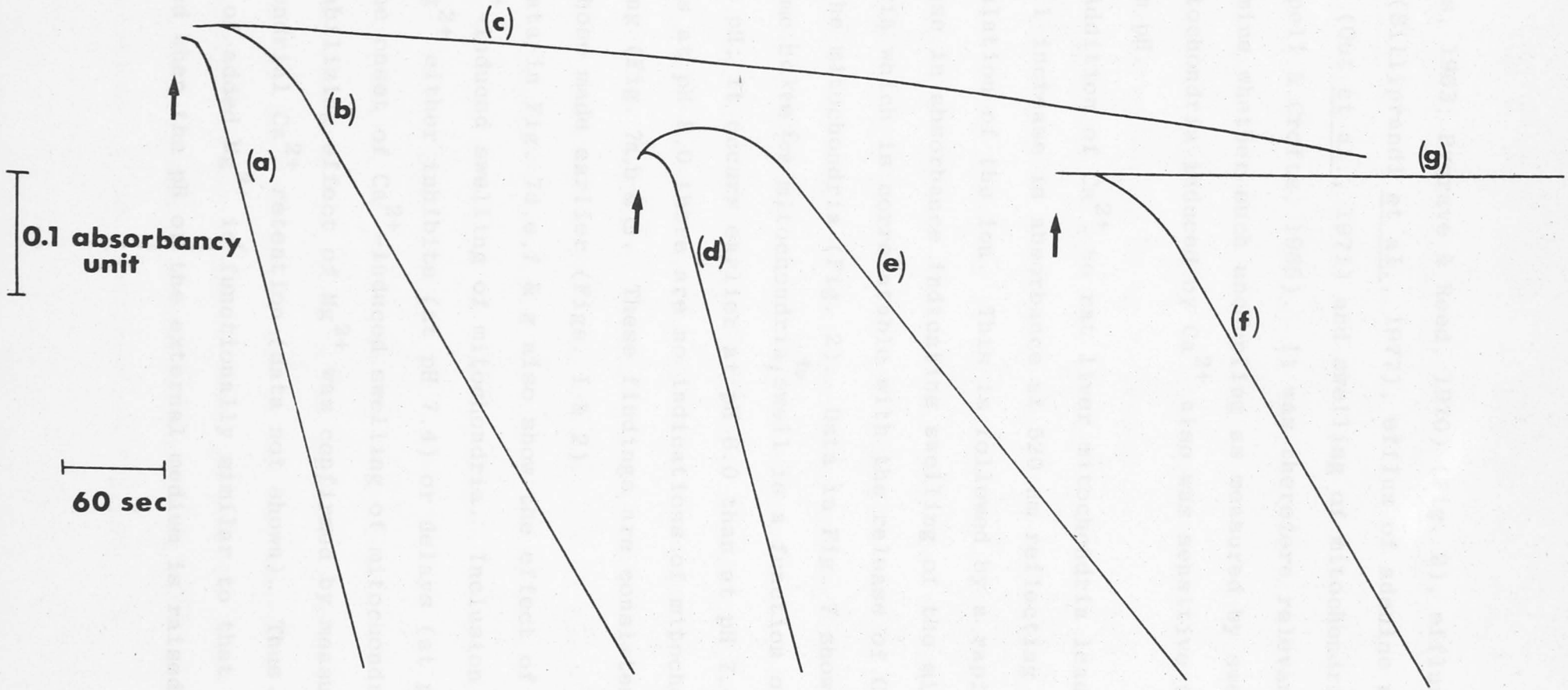
Valinomycin-induced Ca^{2+} uptake into respiration-inhibited mitochondria can be measured in the presence of low external concentrations of K^+ (Scarpa & Azzone, 1970). A study of the effect of the medium pH on valinomycin-induced Ca^{2+} uptake revealed a similar pH dependence of the initial rate of Ca^{2+} uptake to that observed in respiring mitochondria. i.e. the initial rate of Ca^{2+} transport was lower at high H^+ concentrations and higher at low H^+ concentrations (data not shown; Scarpa & Azzone, 1970). The magnitude of the effect of pH in respiration-inhibited mitochondria was lower than in respiring mitochondria.

Effect of medium pH and Mg^{2+} ions on Ca^{2+}-induced swelling of mitochondria

Ca^{2+}-induced uncoupling of rat liver mitochondria, first described by Lehninger (1949), is associated with an increase in the respiratory rate (Rossi & Lehninger, 1964) (Fig. 1), inability of mitochondria to retain Ca^{2+}

Fig. 7. Swelling of mitochondria

Conditions were as in Fig. 1. 100 μM-$CaCl_2$ was added as indicated by the arrow. The pH of the medium was 6.0 (a,d & e), 7.4 (b,f & g) or 8.0 (c). $MgCl_2$ (2 mM) was present in e and g.


(a)
(b)
(c)
(d)
(e)
(f)
(g)
0.1 absorbancy unit
60 sec

(Saris, 1963; Bygrave & Reed, 1970) (Fig. 2), efflux of Mg^{2+} (Silliprandi et al., 1977), efflux of adenine nucleotides (Out et al., 1971) and swelling of mitochondria (Chappell & Crofts, 1965). It was therefore relevant to determine whether such uncoupling as measured by swelling of mitochondria induced by Ca^{2+} also was sensitive to the medium pH.

Addition of Ca^{2+} to rat liver mitochondria leads to a small increase in absorbance at 520 nm reflecting accumulation of the ion. This is followed by a rapid decrease in absorbance indicating swelling of the mitochondria which is correlatable with the release of Ca^{2+} from the mitochondria (Fig. 2). Data in Fig. 7 show that the time taken for mitochondria to swell is a function of the medium pH; it occurs earlier at pH 6.0 than at pH 7.4, whereas at pH 8.0 there are no indications of mitochondrial swelling (Fig. 7a,b & c). These findings are consistent with those made earlier (Figs. 1 & 2).

Data in Fig. 7d,e,f & g also show the effect of Mg^{2+} on Ca^{2+}-induced swelling of mitochondria. Inclusion of 2 mM Mg^{2+} either inhibits (at pH 7.4) or delays (at pH 6.0) the onset of Ca^{2+}-induced swelling of mitochondria. The stabilizing effect of Mg^{2+} was confirmed by measuring mitochondrial Ca^{2+} retention (data not shown). Thus the effect of added Mg^{2+} is functionally similar to that observed when the pH of the external medium is raised.

Influence of medium pH on Pi transport by rat liver mitochondria

It could be argued a priori that the increase in initial rate of Ca^{2+} transport at alkaline pH values is attributable to an increased rate of Pi translocation at an alkaline pH (Fig. 5b). We therefore examined directly the effect of medium pH on Pi transport.

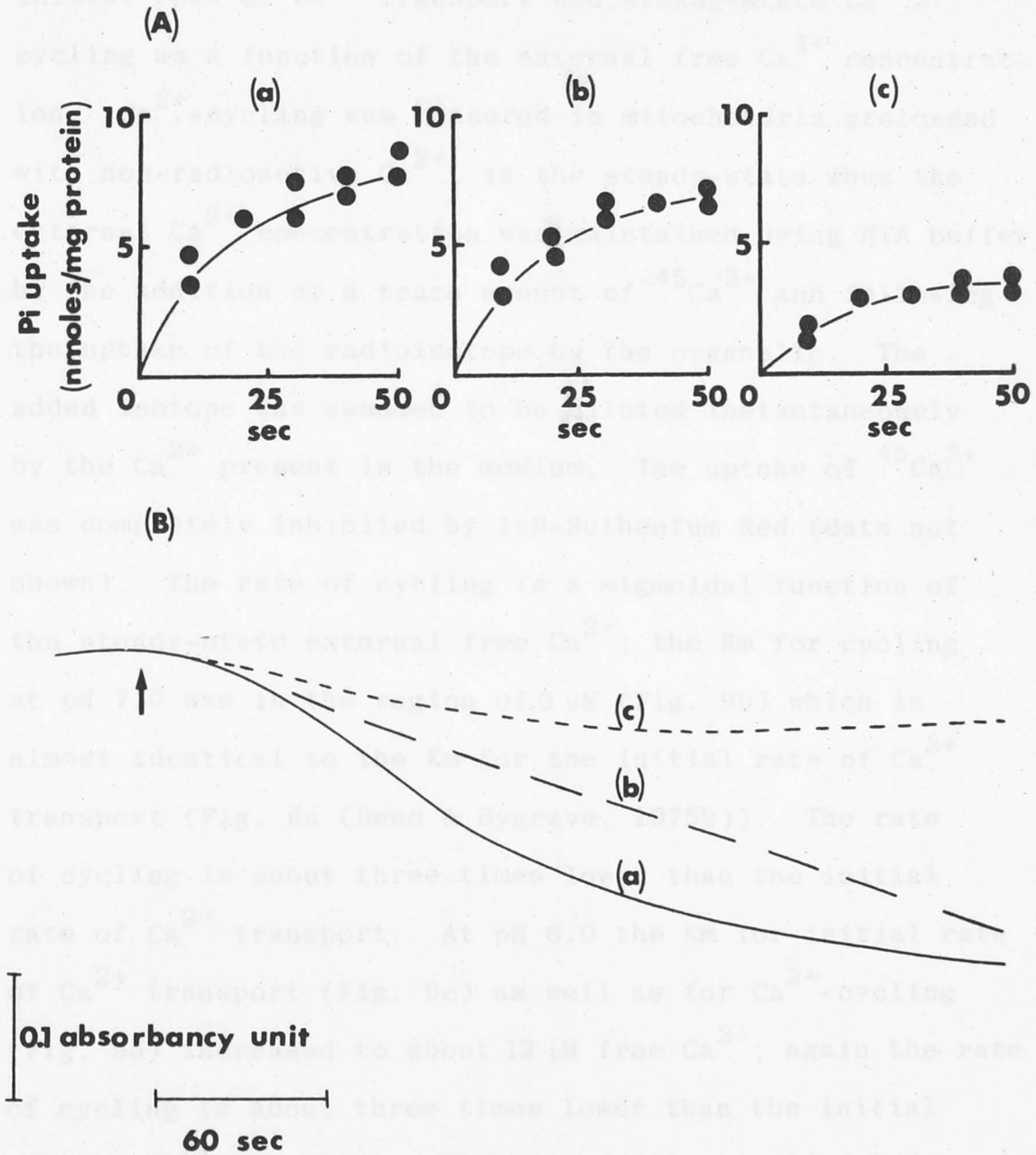
Data in Fig. 8 show the effect of varying the medium pH on the transport of Pi into rat liver mitochondria measured directly either by following the accumulation of chemical Pi in the mitochondria (Fig. 8A) or indirectly by following the swelling of the organelle in the presence of NH_4Cl (Fig. 8B). In both instances and in both LiCl-containing (Fig. 8A) as well as in sucrose-containing media (Fig. 8B), Pi transport shows a marked pH dependence. The rate of transport is higher at pH 6.0 than at pH 7.0 or 8.0. This finding is similar to that obtained by Freitag & Kadenbach (1978) who showed that at low Pi concentrations, Pi transport is higher in an acidic medium whereas at higher Pi concentrations, Pi transport is lower in the same medium.

This pattern of pH dependence of Pi transport, together with the fact that Ca^{2+} transport is stimulated by Pi to the same extent at all pH values, suggests that the transport of the permeant anion is not the rate-limiting step in the initial rate influx of Ca^{2+} under the conditions of the experiment in Fig. 5b.

Fig. 8. Effect of medium pH on Pi transport

Fig. A: Pi transport was measured as described in the Experimental Section. The reaction medium contained 150 mM-LiCl, 0.5 mM-KCl, 5.0 mM-Hepes and 5.0 mM-succinate. Temperature 5°C. The pH was 6.0 (a); 7.0 (b) or 8.0 (c).

Fig. B: Mitochondria (0.4 mg/ml) were incubated in the medium described in Fig. 4. At the points indicated by the arrow, 20 mM each of NH_4Cl and KH_2PO_4 was added. Swelling of mitochondria was followed as described in the Experimental Section. The pH was 6.0 (a); 7.0 (b) and 8.0 (c).


(A)
(a)
(b)
(c)
Pi uptake
(nmoles/mg protein)
10
5
0
25
50
sec
(B)
(c)
(b)
(a)
0.1 absorbancy unit
60 sec

Effect of pH on the initial rate of Ca^{2+} transport and on the steady-state Ca^{2+}-cycling

Data in Fig. 9 show the effect of medium pH on the initial rate of Ca^{2+} transport and steady-state Ca^{2+}-cycling as a function of the external free Ca^{2+} concentration. Ca^{2+}-cycling was measured in mitochondria preloaded with non-radioactive Ca^{2+}, in the steady-state when the external Ca^{2+} concentration was maintained using NTA buffer, by the addition of a trace amount of $^{45}Ca^{2+}$ and following the uptake of the radioisotope by the organelle. The added isotope was assumed to be diluted instantaneously by the Ca^{2+} present in the medium. The uptake of $^{45}Ca^{2+}$ was completely inhibited by 1μM-Ruthenium Red (data not shown). The rate of cycling is a sigmoidal function of the steady-state external free Ca^{2+}; the Km for cycling at pH 7.0 was in the region of 3 μM (Fig. 9b) which is almost identical to the Km for the initial rate of Ca^{2+} transport (Fig. 9a (Reed & Bygrave, 1975b)). The rate of cycling is about three times lower than the initial rate of Ca^{2+} transport. At pH 6.0 the Km for initial rate of Ca^{2+} transport (Fig. 9c) as well as for Ca^{2+}-cycling (Fig. 9d) increased to about 12 μM free Ca^{2+}; again the rate of cycling is about three times lower than the initial rate of Ca^{2+} transport. Further experiments revealed that the Vmax for the initial rate of Ca^{2+} transport and Ca^{2+}-cycling is not significantly affected by the pH of the medium. This indicates a competetive type of inhibition by H^{+} in accordance with the conclusion reached

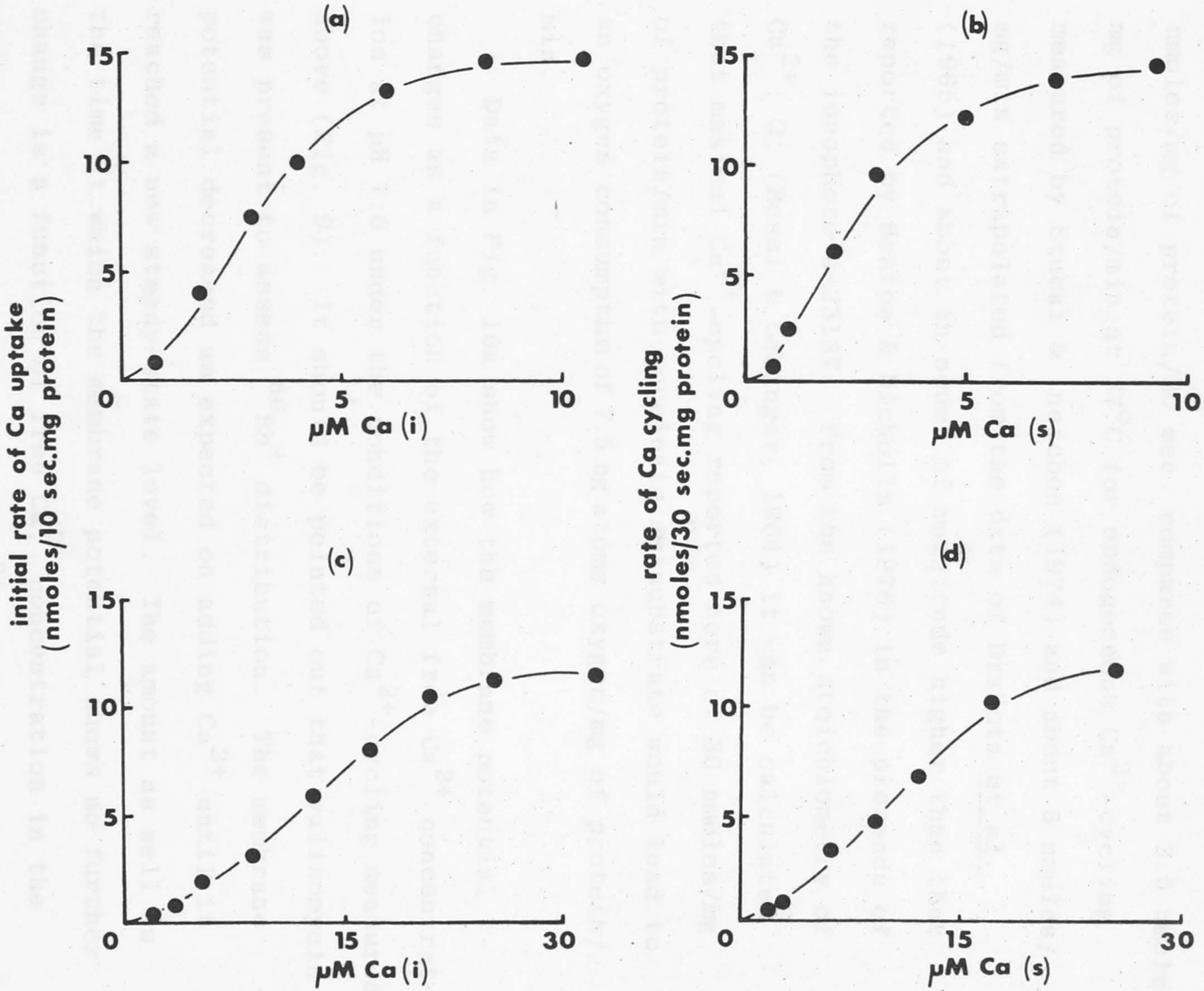
Fig. 9. Calcium-cycling

Mitochondria (1 mg/ml) were preincubated for 1 min at 14°C in a medium containing 230 mM-sucrose, 5 mM-Hepes, 5 mM-succinate, 5 μM-rotenone and 10 mM-NTA. Varying amounts of $^{45}Ca^{2+}$ were added to initiate Ca^{2+} transport. The transport was terminated by rapid filtration of an aliquot followed by washing with EGTA-containing solution to remove Ca^{2+} that was not transported (Reed & Bygrave, 1974a, 1975b). The filter papers were dissolved in scintillation fluid and counted for radioactivity as described in the Experimental Section. Samples were taken at 10 sec and 5 min to calculate the initial rate and steady-state Ca^{2+} transport, respectively. Duplicate incubations were carried out with non-radioactive Ca^{2+} and 5 min after addition of Ca^{2+}, a trace amount of $^{45}Ca^{2+}$ (400,000 cpm) was added to initiate Ca^{2+}-cycling and aliquots of the reaction mixture was filtered and washed as described above.

The pH of the incubation medium in traces a & b was 7.0 and in c & d was 6.0. Initial rate of Ca^{2+} transport traces a & c; Ca^{2+}-cycling traces b & d.

Ca(i) = initial free Ca^{2+} concentration in the medium

Ca(s) = steady-state free Ca^{2+} concentration in the medium


(a)
(b)
(c)
(d)
initial rate of Ca uptake
(nmoles/10 sec.mg protein)
rate of Ca cycling
(nmoles/30 sec.mg protein)
15
10
5
0
5
10
15
30
μM Ca (i)
μM Ca (s)

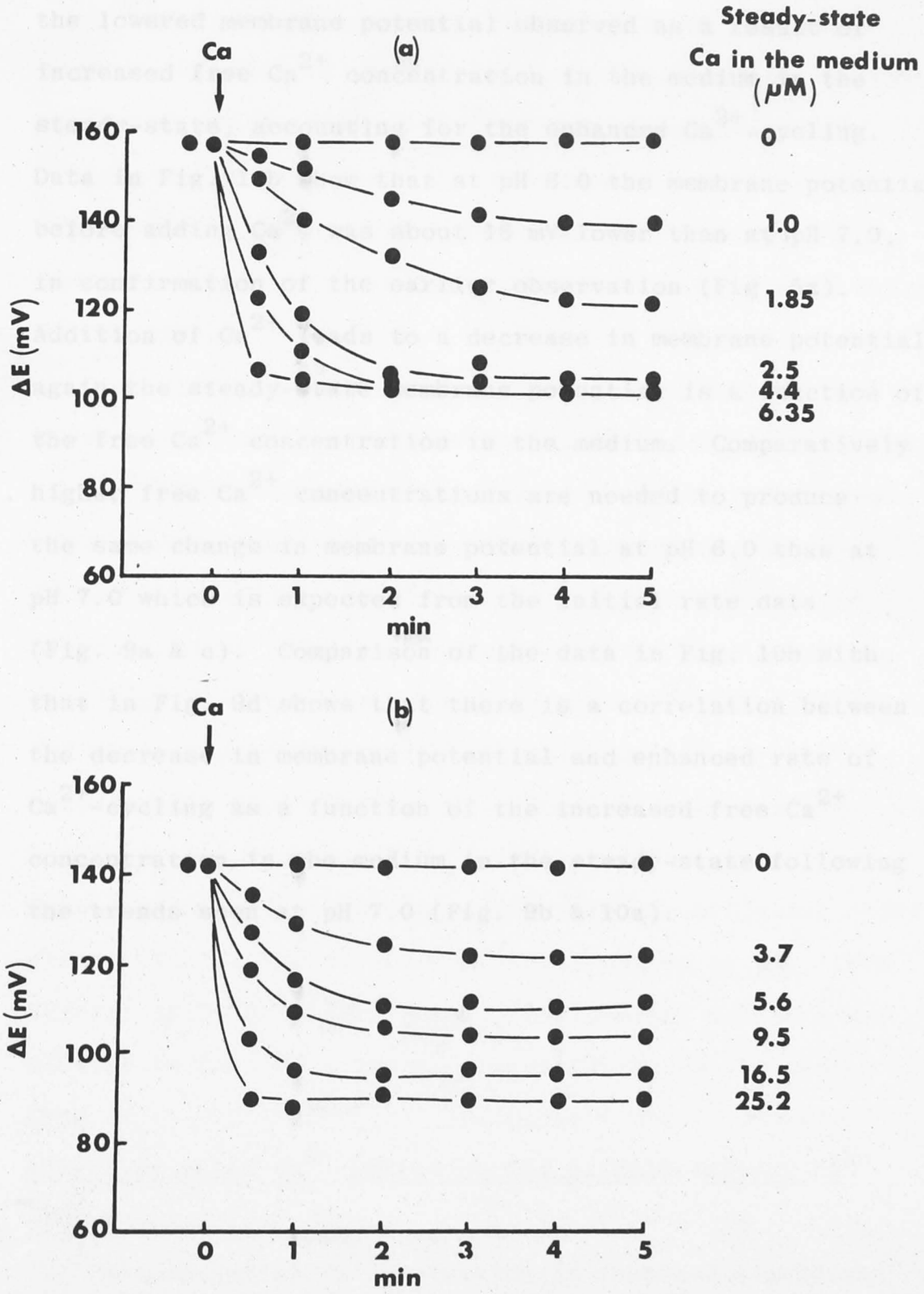
by Scarpa & Azzone (1970) for a competetive type of inhibition of valinomycin-induced Ca^{2+} transport. The maximal rate of cycling measured here of about 14-16 nmoles/mg of protein/30 sec. compares with about 3.5 nmoles/mg of protein/min at 37°C for endogeneous Ca^{2+}-cycling measured by Stucki & Ineichen (1974) and about 8 nmoles/mg/min extrapolated from the data of Drahota et al. (1965) and about an order of magnitude higher than that reported by Heaton & Nicholls (1976) in the presence of the ionophore A-23187. From the known stoichiometry of Ca^{2+}: O, (Rossi & Lehinger, 1964) it can be calculated that maximal Ca^{2+}-cycling reported here of 30 nmoles/mg of protein/min with succinate as substrate would lead to an oxygen consumption of 7.5 ng atoms oxygen/mg of protein/min.

Data in Fig. 10a show how the membrane potential changes as a function of the external free Ca^{2+} concentration at pH 7.0 under the conditions of Ca^{2+}-cycling measured above (Fig. 9). It should be pointed out that valinomycin was present to assess $^{86}Rb^{+}$ distribution. The membrane potential decreased as expected on adding Ca^{2+} until it reached a new steady-state level. The amount as well as the time at which the membrane potential shows no further change is a function of free Ca^{2+} concentration in the medium. The increased rate of Ca^{2+}-cycling as a function of the external free Ca^{2+} concentration (Fig. 9b) is interpreted as resulting from the decrease in membrane potential observed (Fig. 10a). The strain needed to

Fig. 10. The membrane potential and the steady-state Ca^{2+} concentration

Mitochondria (1 mg/ml) were preincubated for 2 min at 14°C in the medium described in Fig. 9 in the presence of 0.5 μM-valinomycin 0.5 mM-KCl, $^{86}RbCl$ and [U-^{14}C] sucrose. Varying amounts of Ca^{2+} were added at time zero in the Fig. Membrane potential was determined as described in the Experimental Section. A duplicate incubation with $^{45}Ca^{2+}$ was used to determine the steady-state free Ca^{2+} concentration (Ca) in the medium. The pH of the reaction medium were 7.0 (a) and 6.0 (b).

ΔE = membrane potential


Steady-state
Ca in the medium
(μM)
Ca
(a)
160
140
120
100
80
60
ΔE (mV)
0
1.0
1.85
2.5
4.4
6.35
0
1
2
3
4
5
min
Ca
(b)
160
140
120
100
80
60
ΔE (mV)
0
3.7
5.6
9.5
16.5
25.2
0
1
2
3
4
5
min

pump Ca^{2+} from the matrix to the medium is decreased by the lowered membrane potential observed as a result of increased free Ca^{2+} concentration in the medium in the steady-state, accounting for the enhanced Ca^{2+}-cycling. Data in Fig. 10b show that at pH 6.0 the membrane potential before adding Ca^{2+} was about 16 mV lower than at pH 7.0, in confirmation of the earlier observation (Fig. 5a). Addition of Ca^{2+} leads to a decrease in membrane potential; again the steady-state membrane potential is a function of the free Ca^{2+} concentration in the medium. Comparatively higher free Ca^{2+} concentrations are needed to produce the same change in membrane potential at pH 6.0 than at pH 7.0 which is expected from the initial rate data (Fig. 9a & c). Comparison of the data in Fig. 10b with that in Fig. 9d shows that there is a correlation between the decrease in membrane potential and enhanced rate of Ca^{2+}-cycling as a function of the increased free Ca^{2+} concentration in the medium in the steady-state following the trends seen at pH 7.0 (Fig. 9b & 10a).

DISCUSSION

The set of observations described in this chapter provide information that bears directly on aspects of the mechanism and control of Ca^{2+} fluxes across the inner mitochondrial membrane. The information was achieved by systematically measuring a number of Ca^{2+}-dependent, energy-linked reactions in mitochondria suspended in media of varying pH.

The first point revealed by this work is that rat liver mitochondria are resistant to the uncoupling action of Ca^{2+} when suspended in an alkaline medium. Thus these mitochondria exhibited controlled 'respiratory jumps' (Rossi & Lehninger, 1964) when pulsed with Ca^{2+} in the presence of Pi (Fig. 1), did not exhibit large amplitude swelling following the accumulation of Ca^{2+} (Fig. 7), and were able to retain the accumulated Ca^{2+} for considerable periods (Fig. 2). Some evidence was provided too that the presence of Mg^{2+} in the medium, which is known to 'stabilize' mitochondria (Leblanc & Clauser, 1974; Binet & Volfin, 1975; Sordahl, 1975; Hunter et al., 1976; Harris, 1979; Lehninger et al., 1967) might act in a way similar to that of a low H^{+} concentration in the medium (Fig. 7).

Effect of pH on Ca^{2+} retention and on EGTA-induced Ca^{2+} efflux

Mitochondrial Ca^{2+} retention is enhanced significantly when the pH of the suspending medium is increased from 7.0

to 8.0 (Fig. 2). Because the membrane potential (Fig. 5a), initial rates of Ca^{2+} transport and rates of ADP-stimulated and resting respiration (data not shown) are similar at pH 7.0 and 8.0, these parameters most likely are not critical in promoting Ca^{2+} retention. By contrast, the transmembrane pH gradient (Fig. 5a), initial rates of Pi transport (Fig. 8a,b) and EGTA-induced Ca^{2+} efflux (Fig. 4) are considerably less at pH 8.0 than at pH 7.0.

Several of the experiments described in this report raise the possibility that the concentration of free (ionic) Ca^{2+} in the EGTA-inaccessible compartment in rat liver mitochondria might be lower at an alkaline pH despite the fact that the total amount of Ca^{2+} accumulated was the same at pH 7.0 and pH 8.0 (Fig. 3). This may be an important factor contributing to the ability of the mitochondria to retain Ca^{2+} at pH 8.0. The evidence leading to this conclusion is admittedly indirect and based largely on the observed response of the membrane potential to the movements of Ca^{2+} into the EGTA-inaccessible space at varying pH values (Figs. 2,3).

If it is assumed that the free species only of accumulated Ca^{2+} perturbs the membrane potential, then it can be argued that where the membrane potential is least perturbed following the accumulation of Ca^{2+}, the concentration of free Ca^{2+} is least. On this basis it could be concluded that the concentration of free Ca^{2+}

in the mitochondrial matrix would be less at an alkaline pH (Figs. 2,3). Further support for this argument arises from the observation that the rate of EGTA-induced Ca^{2+} efflux from rat liver mitochondria, which should reflect the availability of internal free Ca^{2+}, is lower in media maintained at higher pH values (Fig. 4).

The lower membrane potential in the steady-state before and after addition of Ca^{2+} at pH 6.0 than at pH 7.0 (cf. Fig. 3a and b; Fig. 10a and b), could also account for the enhanced rate of EGTA-induced efflux observed at pH 6.0 than at pH 7.0 (Fig. 4).

The maintainance of a lower free Ca^{2+} concentration in the mitochondrial matrix at an alkaline pH environment could presumably result from a greater availability of internal Ca^{2+}-binding sites. The physico-chemical nature of these sites is largely unknown but they would include in all probability oxyanion-type ligands (Williams, 1970, 1974) on proteins and phospholipids, metabolites and substrates like adenine nucleotides, Pi and carboxylic acids, all of which would be more likely to exist in the dissociated state at an alkaline pH and therefore be more amenable for binding by Ca^{2+}.

Effect of pH on Ca^{2+} influx

The effect of pH on the initial rate influx of Ca^{2+} seen here is similar to that reported previously (Reed & Bygrave, 1975b). The initial rate increased approx. 4-fold with increasing pH from 6.0 until it reached a maximum at approx. pH 7.5. Since the protonmotive force remained

constant in this pH range (Fig. 5), it is difficult to envisage that the initial rate of Ca^{2+} transport is limited by the protonmotive force. This is especially so because the presence of Pi can induce an increase in the membrane potential at pH 6.0 to values slightly greater than those observed at pH 8.0 in the absence of Pi. Yet the initial rate of Ca^{2+} transport at pH 6.0 in the presence of Pi is considerably less than in its absence at pH 8.0. These findings regarding the role of the membrane potential on the initial rate of Ca^{2+} transport are in full agreement with the conclusions reached in earlier reports from this laboratory (see e.g. Bygrave et al., 1978a).

Although the initial rate of Pi transport showed a marked pH-dependence, being higher at an acidic pH (Fig. 8), its ability to stimulate the initial rate of Ca^{2+} transport was similar at all pH values (Fig. 5b), indicating that Pi transport in these experiments is not a rate-limiting factor in the stimulation of the initial rate of Ca^{2+} transport. It has been previously suggested that anions stimulate Ca^{2+} transport by enhancing the dissociation of Ca^{2+} from the Ca^{2+} carrier on the matrix side of the inner membrane (Reed & Bygrave, 1975b).

Steady-state Ca^{2+}-cycling in mitochondria

The present data do not permit conclusions to be drawn about the effect of pH on steady-state Ca^{2+}-cycling, at least under the conditions carried out here. This arises because as seen in Fig. 9b and d, the rate of Ca^{2+}-

cycling is related to the concentration of external free Ca^{2+}; at pH 7.0 maximal rates of cycling are seen at approx. 10 μM free Ca^{2+} whereas at pH 6.0 it is seen at approx. 25 μM free Ca^{2+}. This situation in turn may arise out of the difference in the Km for the initial rate of Ca^{2+} transport (Fig. 9a and c) at the two pH values.

The increase in Ca^{2+}-cycling that occurs as the external free Ca^{2+} concentration is increased (Fig. 9b and d) can be considered in light of reports that the accumulation ratios of monovalent and divalent cations are not in electrochemical equilibrium with each other (Puskin et al., 1976; Azzone et al., 1977; Pozzan et al., 1977). When considered against values of the membrane potential across the inner mitochondrial membrane (greater than 150 mV negative inside) this supports the suggestion of a passive influx mechanism for Ca^{2+}, driven by the membrane potential negative inside, and an active efflux mechanism operating against the membrane potential (Gunter et al., 1978). If this is so then the lower the membrane potential the higher will be the rate of Ca^{2+} efflux from the mitochondria since the energy needed to pump the ion out of the mitochondria would be less than when the membrane has a higher potential across it. Hence one might well expect to see an increase in rate of Ca^{2+}-cycling with a decrease in membrane potential especially as the membrane potential appears not to limit the initial rate of Ca^{2+} influx (Fig. 5). This would be consistent also with the enhanced rate of Ca^{2+}-cycling that occurs

concomitant with the increase in external free Ca^{2+} concentration associated with a decrease in the membrane potential (Fig. 10a and b).

Physiological implications

As pointed out in the Introduction, glucagon (Titheradge & Coore, 1976; Halestrap, 1978; Prpic et al., 1978) and α-adrenergic agonists (Taylor et al., 1980) administered to rats in vivo induce an increase in the mitochondrial transmembrane pH of approx. 10 mV or about 0.15 pH units. This leads us to conclude that as far as can be presently judged, the increase in transmembrane pH seen after hormone treatment of rat liver mitochondria in situ is not in itself sufficient to account for the increase in Ca^{2+} retention in those mitochondria and that some other factor(s) as well must be involved (e.g. Prpic et al., 1978).

Finally while the experimental approach and arguments made here may give the impression that we are dealing only with changes in the bulk phase pH, we should point out the possibility that these most likely are in equilibrium with pH changes occurring in microenvironments in or on the inner mitochondrial membrane (Williams, 1969; Robertson & Boardman, 1975) and that it is within these environments that the true loci exist where the pH changes are expressing their influence on the inward and outward fluxes of Ca^{2+} described here. The possible existence of steep gradients of Ca^{2+} and H^{+} in specific microenvironments proximal to the inner mitochondrial membrane (see e.g.

Bygrave, 1978a), as mooted for other metabolites in mitochondria (see e.g. Duszynski et al., 1978), may well facilitate the means by which an efficient interaction between H^+ concentrations and Ca^{2+} fluxes will occur.

CHAPTER 7

RUTHENIUM RED-SENSITIVE AND-INSENSITIVE RELEASE OF CALCIUM BY MITOCHONDRIA ISOLATED FROM RAT LIVER AND RAT HEART

RUTHENIUM RED-SENSITIVE AND-INSENSITIVE RELEASE OF CALCIUM BY MITOCHONDRIA ISOLATED FROM RAT LIVER AND RAT HEART

INTRODUCTION

Mitochondria accumulate Ca^{2+} in an energy-dependent manner, in response to the membrane potential, negative inside, generated either by respiration or ATP hydrolysis (Lehninger, 1970; Bygrave, 1977; Mela, 1977; Carafoli & Crompton, 1978). In order for this uptake process to have any physiological significance in regulating cell Ca^{2+}, the accumulated Ca^{2+} should be available for rapid release. Drahota *et al.* (1965) reported that Ca^{2+} was retained by the mitochondria not as a result of irreversible sequestration of the ion by the organelle, but as a result of a steady-state in which the release of Ca^{2+} down the concentration gradient was compensated by the energy-linked uptake process. In support of this idea, uncouplers or inhibitors of oxidative phosphorylation induce a net release of Ca^{2+} from the mitochondria (Lehninger *et al.*, 1967; Carafoli, 1974; Bygrave, 1977).

The search for physiological agents able to specifically induce a release of Ca^{2+} from mitochondria has led to the identification of (a) Pi which presumably effects release by promoting uncoupling (Lehninger *et al.*, 1967), (b) the glycolytic intermediate, phosphoenol-pyruvate (Chudapongse & Haugaard, 1973; Peng *et al.*, 1974; Sul *et al.*, 1976) which effects release by exchanging with adenine

nucleotides with consequent uncoupling (Ross et al., 1978), (c) palmitoyl CoA (Asimakis & Sordahl, 1977) and (d) Na^+ in tissues like heart and brain but not in liver or kidney (Crompton et al., 1976,1977,1978). Recently Lehninger et al. (1978b) reported that the redox state of pyridine nucleotides also may control Ca^{2+} transport by mitochondria. They showed that when mitochondrial pyridine nucleotides were in the oxidized state, Ca^{2+} is released from the organelle.

Sordahl (1975) showed that the release of Ca^{2+} induced by A23187 was not sensitive to Ruthenium Red and suggested the existence of two pathways for movement of Ca^{2+} across the inner mitochondrial membrane, one involving entry of the ion, the other its exit. It is now known that the efflux of Ca^{2+} induced by Na^+ (Carafoli & Crompton, 1978), by palmitoyl CoA (Asimakis & Sordahl, 1977) and by the oxidized state of pyridine nucleotides (Lehninger et al., 1978b) is not sensitive to Ruthenium Red. Also, the release induced by the above agents appears not to involve mitochondrial damage. On the other hand Reed & Bygrave (1974b) showed that EGTA induced a release of Ca^{2+} from mitochondria that was inhibited by Ruthenium Red, a finding that since has been confirmed (Pozzan et al., 1977).

Recent experiments in this laboratory have shown that the EGTA-induced efflux of Ca^{2+} from mitochondria is sensitive to the pH of the incubation medium (Chapter 6 Fig. 4) and is altered following perfusion of rat liver

with glucagon and with α-adrenergic agonists (Taylor et al., 1980). This prompted us to investigate further the mechanism of EGTA-induced Ca^{2+} efflux and is described in this chapter.

RESULTS

EGTA-induced Ca^{2+} efflux from rat liver and rat heart mitochondria

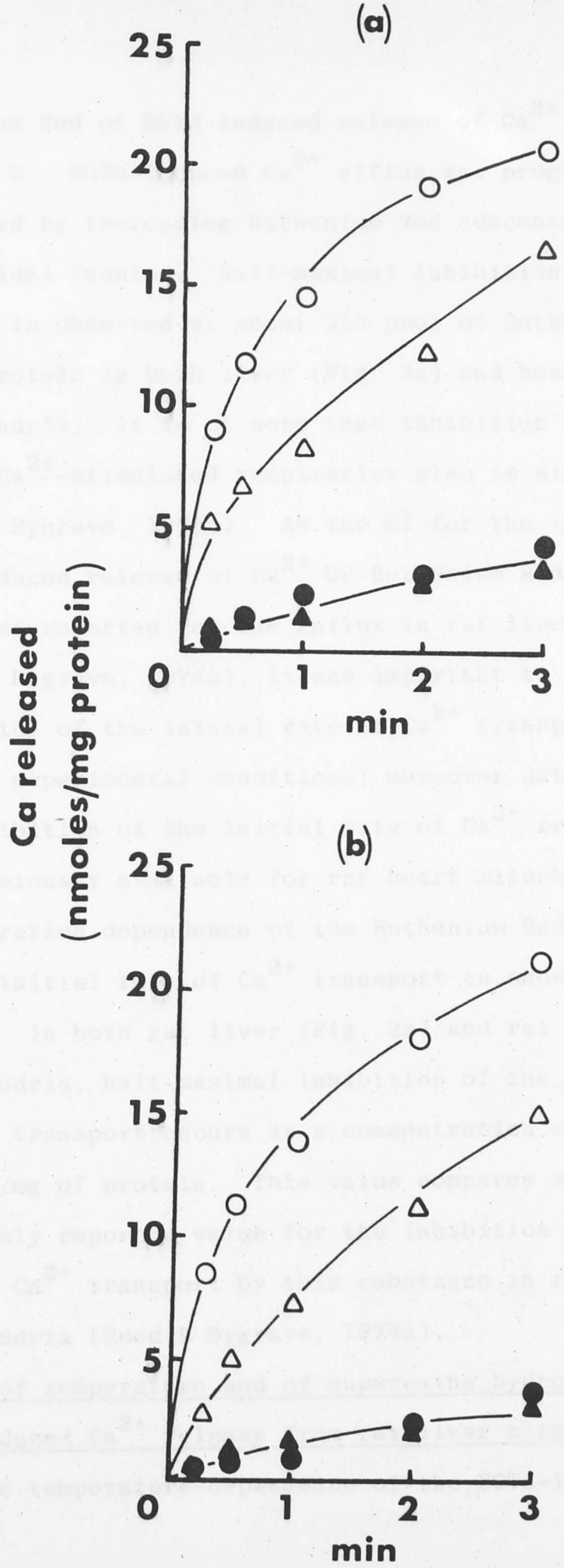
Data in Fig. 1 show the efflux of Ca^{2+} induced by EGTA from rat liver and rat heart mitochondria. In this experiment mitochondria respiring in the presence of succinate were allowed to accumulate Ca^{2+} for about 4 min; by that time a steady-state was achieved in which no further net accumulation of the ion occurred. Generally both types of mitochondria accumulated about 80% of the added Ca^{2+}. The efflux of Ca^{2+} induced by adding 2 mM-EGTA, was rapid from both liver (Fig. 1a) and heart (Fig. 1b) mitochondria. The rate of Ca^{2+} release as well as the amount of Ca^{2+} released were of similar magnitude in both liver and heart mitochondria. KCl inhibited the rate of Ca^{2+} release by about 40% in both types of mitochondria. The simultaneous addition of 1 μM-Ruthenium Red with EGTA largely abolished the EGTA-induced release of Ca^{2+} in both types of mitochondria and in both sucrose- and KCl-containing medium. This finding confirms the earlier observation from this laboratory (Reed & Bygrave, 1974b) and shows that Ruthenium Red-sensitive Ca^{2+} release induced by EGTA occurs also in rat heart mitochondria.

Effect of Ruthenium Red on EGTA-induced Ca^{2+} efflux and the initial rate of Ca^{2+} transport in rat liver and rat heart mitochondria

The concentration dependence of the inhibition by

Fig. 1. EGTA-induced Ca^{2+} efflux from rat liver and rat heart mitochondria.

The reaction medium contained in a total volume of 2.0 ml either 230 mM-sucrose (○,●) or 150 mM-KCl (△,▲) and 5 mM-succinate, 5 mM-Hepes/Tris (pH 7.4) and 5 μM-rotenone. Mitochondria from rat liver (a) or rat heart (b) at 1 mg/ml were preincubated for 1 min at 25°C. 100 μM-$CaCl_2$ (containing 0.5 μCi $^{45}Ca^{2+}$) was added to initiate Ca^{2+} transport. After 4 min 2 mM-EGTA with (●,▲) or without (○,△) 1 μM-Ruthenium Red was added at time zero in the Fig. Samples were removed at the times shown for measurement of Ca^{2+} transport as described in the Experimental Section.


(a)
25
20
15
10
5
0
1
2
3
min
Ca released
(nmoles/mg protein)
(b)
25
20
15
10
5
0
1
2
3
min

Ruthenium Red of EGTA-induced release of Ca^{2+} is shown in Fig. 2. EGTA-induced Ca^{2+} efflux was progressively inhibited by increasing Ruthenium Red concentrations in a sigmoidal fashion. Half-maximal inhibition of Ca^{2+} release is observed at about 350 pmol of Ruthenium Red/ mg of protein in both liver (Fig. 2a) and heart (Fig. 2b) mitochondria. It is of note that inhibition by Ruthenium Red of Ca^{2+}-stimulated respiration also is sigmoidal (Reed & Bygrave, 1974a). As the Ki for the inhibition of EGTA-induced release of Ca^{2+} by Ruthenium Red was higher than that reported for the influx in rat liver mitochondria (Reed & Bygrave, 1974a), it was important to compare the inhibition of the initial rate of Ca^{2+} transport under the present experimental conditions; moreover data for Ruthenium Red inhibition of the initial rate of Ca^{2+} transport was not previously available for rat heart mitochondria. The concentration dependence of the Ruthenium Red inhibition of the initial rate of Ca^{2+} transport is shown also in Fig. 2. In both rat liver (Fig. 2a) and rat heart (Fig. 2b) mitochondria, half-maximal inhibition of the initial rate of Ca^{2+} transport occurs at a concentration of about 50 pmol/mg of protein. This value compares well with the previously reported value for the inhibition of the initial rate of Ca^{2+} transport by this substance in rat liver mitochondria (Reed & Bygrave, 1974a).

Effect of temperature and of nupercaine hydrochloride on EGTA-induced Ca^{2+} release from rat liver mitochondria.

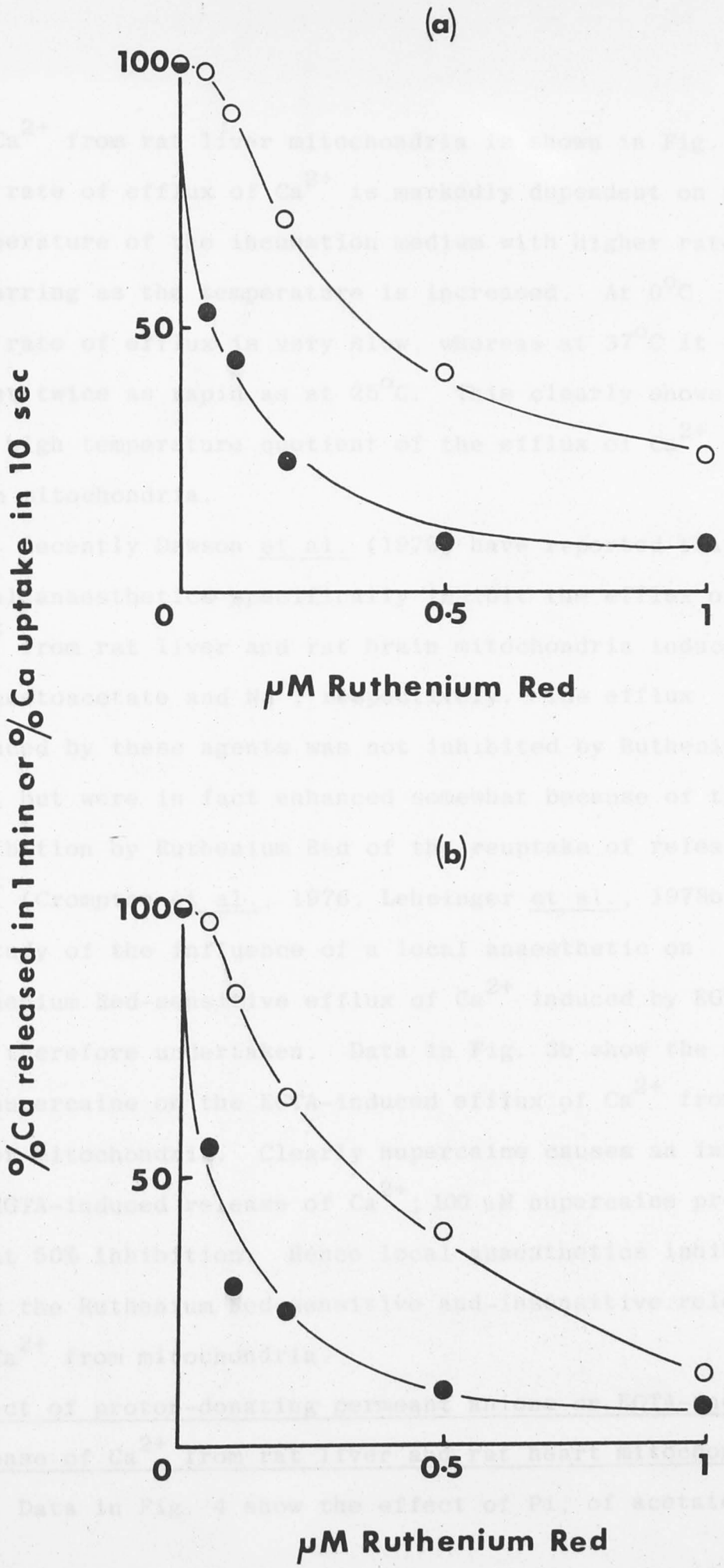
The temperature-dependence of the EGTA-induced efflux

Fig. 2. Concentration dependence of Ruthenium Red inhibition of EGTA-induced Ca^{2+} efflux and initial rate of Ca^{2+} transport in rat liver and rat heart mitochondria

The sucrose medium of Fig. 1 was used and the conditions were identical to that in Fig. 1. The amount of Ruthenium Red added with EGTA was varied as shown in the Fig. The initial rate of Ca^{2+} transport was performed at 12-14°C. Ruthenium Red was added 15 sec before adding Ca^{2+}. Ca^{2+} released in 1 min (○) or Ca^{2+} uptake in 10 sec (●) without Ruthenium Red was equated to 100%.

a-rat liver mitochondria

b-rat heart mitochondria


(a)
100
50
0
0·5
1
μM Ruthenium Red
%Ca released in 1 min or %Ca uptake in 10 sec
(b)
100
50
0
0·5
1
μM Ruthenium Red

of Ca^{2+} from rat liver mitochondria is shown in Fig. 3a. The rate of efflux of Ca^{2+} is markedly dependent on the temperature of the incubation medium with higher rates occurring as the temperature is increased. At $0^{o}C$ the rate of efflux is very slow, whereas at $37^{o}C$ it is about twice as rapid as at $25^{o}C$. This clearly shows the high temperature quotient of the efflux of Ca^{2+} from mitochondria.

Recently Dawson et al. (1979) have reported that local anaesthetics specifically inhibit the efflux of Ca^{2+} from rat liver and rat brain mitochondria induced by acetoacetate and Na^{+}, respectively. The efflux induced by these agents was not inhibited by Ruthenium Red, but were in fact enhanced somewhat because of the inhibition by Ruthenium Red of the reuptake of released Ca^{2+} (Crompton et al., 1976; Lehninger et al., 1978b). A study of the influence of a local anaesthetic on Ruthenium Red-sensitive efflux of Ca^{2+} induced by EGTA was therefore undertaken. Data in Fig. 3b show the effect of nupercaine on the EGTA-induced efflux of Ca^{2+} from rat liver mitochondria. Clearly nupercaine causes an inhibition of EGTA-induced release of Ca^{2+}; 100 μM nupercaine produces about 50% inhibition. Hence local anaesthetics inhibit both the Ruthenium Red-sensitive and-insensitive release of Ca^{2+} from mitochondria.

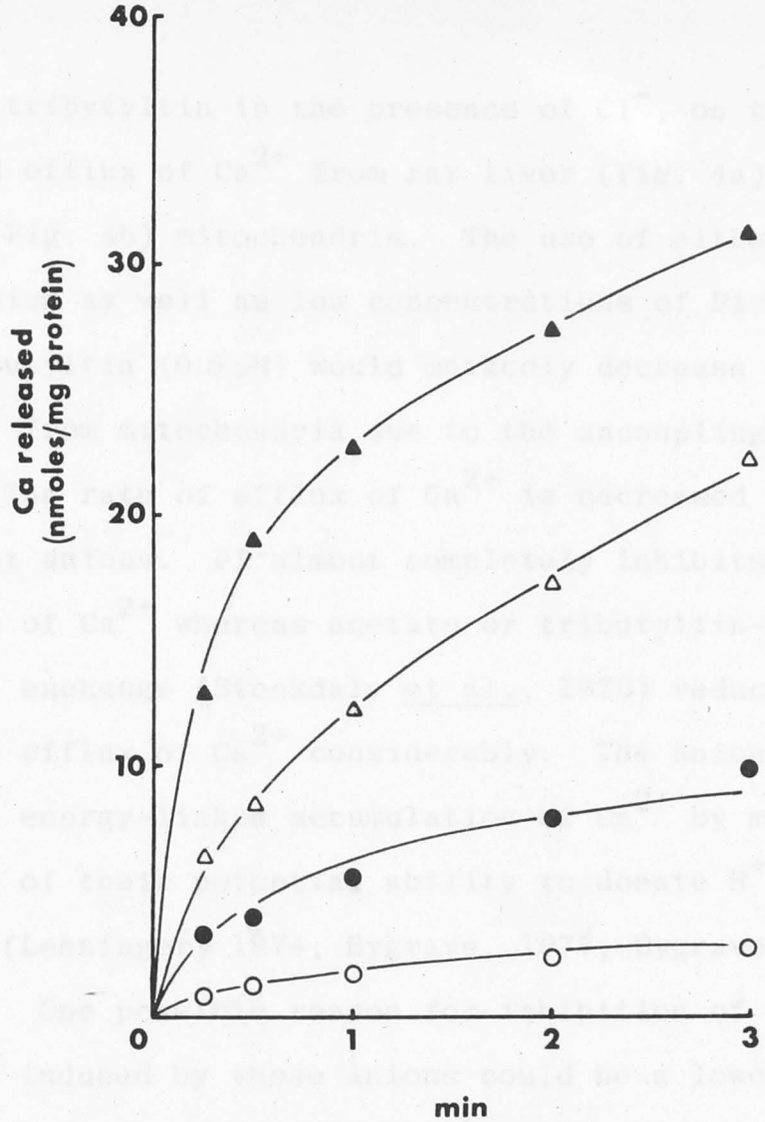
Effect of proton-donating permeant anions on EGTA-induced release of Ca^{2+} from rat liver and rat heart mitochondria

Data in Fig. 4 show the effect of Pi, of acetate,

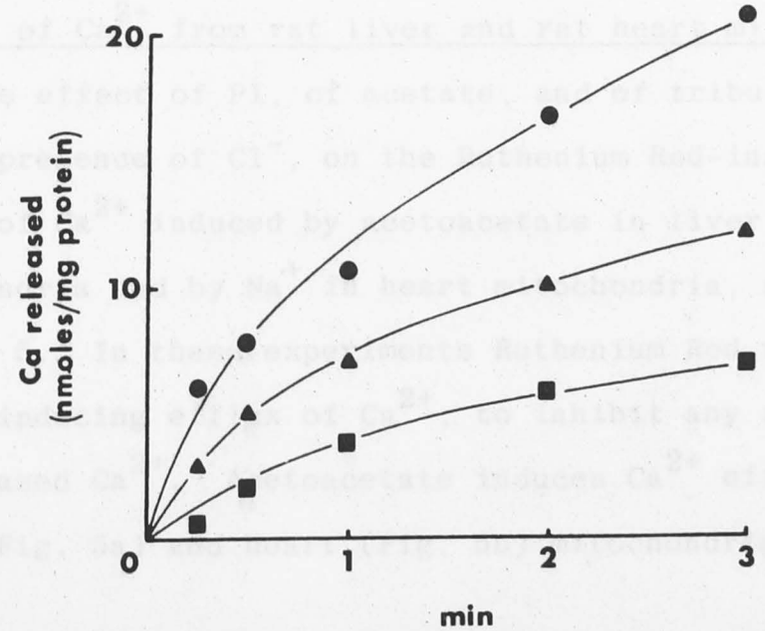
Fig. 3. Effect of temperature and nupercaine hydrochloride on EGTA-induced Ca^{2+} efflux from rat liver mitochondria

Experimental conditions were as in Fig. 1 and the sucrose medium was used. The temperature of the medium in Fig. 3a was $0^{o}C$ (○), $15^{o}C$ (●), $25^{o}C$ (△) or $37^{o}C$ (▲). The amount of nupercaine hydrochloride added in Fig. 3b was 0(●), 100 (▲) and 200 (■) μM and the temperature was $25^{o}C$.

(a)


40
30
20
10
0
1
2
3
Ca released
(nmoles/mg protein)
min

(b)


20
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Ca released
(nmoles/mg protein)
min

and of tribytyltin in the presence of Cl^-, on the EGTA-induced efflux of Ca^{2+} from rat liver (Fig. 4a) and rat heart (Fig. 4b) mitochondria. The use of either LiCl or KCl medium as well as low concentrations of Pi (0.5 mM) or tributyltin (0.5 μM) would markedly decrease any release of Ca^{2+} from mitochondria due to the uncoupling action of Ca^{2+}. The rate of efflux of Ca^{2+} is decreased by the permeant anions. Pi almost completely inhibits the release of Ca^{2+} whereas acetate or tributyltin-mediated Cl^-/OH^- exchange (Stockdale et al., 1970) reduces the rate of efflux of Ca^{2+} considerably. The anions tested support energy-linked accumulation of Ca^{2+} by mitochondria, because of their potential ability to donate H^+ to the matrix (Lehninger, 1974; Bygrave, 1977; Bygrave et al., 1978b). One possible reason for inhibition of Ca^{2+} release induced by these anions could be a lowered free Ca^{2+} concentration in the mitochondria, though other explanations are possible (see Discussion).

Effect of permeant anions on Ruthenium Red-insensitive release of Ca^{2+} from rat liver and rat heart mitochondria.

The effect of Pi, of acetate, and of tributyltin in the presence of Cl^-, on the Ruthenium Red-insensitive efflux of Ca^{2+} induced by acetoacetate in liver and heart mitochondria and by Na^+ in heart mitochondria, are shown in Fig. 5. In these experiments Ruthenium Red was added before inducing efflux of Ca^{2+}, to inhibit any reuptake of released Ca^{2+}. Acetoacetate induces Ca^{2+} efflux in liver (Fig. 5a) and heart (Fig. 5b) mitochondria presumably

Fig. 4. Effect of permeant anions on the Ruthenium Red-sensitive Ca^{2+} efflux from rat liver and rat heart mitochondria

Experimental conditions were as in Fig. 1 except that sucrose was replaced by 150 mM-LiCl (Fig. 4a) and 150 mM-KCl (Fig. 4b) and the temperature was 31°C. 2 mM-EGTA was added 2 min after adding Ca^{2+}.

a - rat liver mitochondria

b - rat heart mitochondria

Control (○), 0.5 mM-Pi (●), 5 mM-acetate (△) and 0.5 μM-tributyltin (▲). The amount of Ca^{2+} accumulated by 2 min ranged from 85 to 92 nmol/mg of mitochondrial protein.

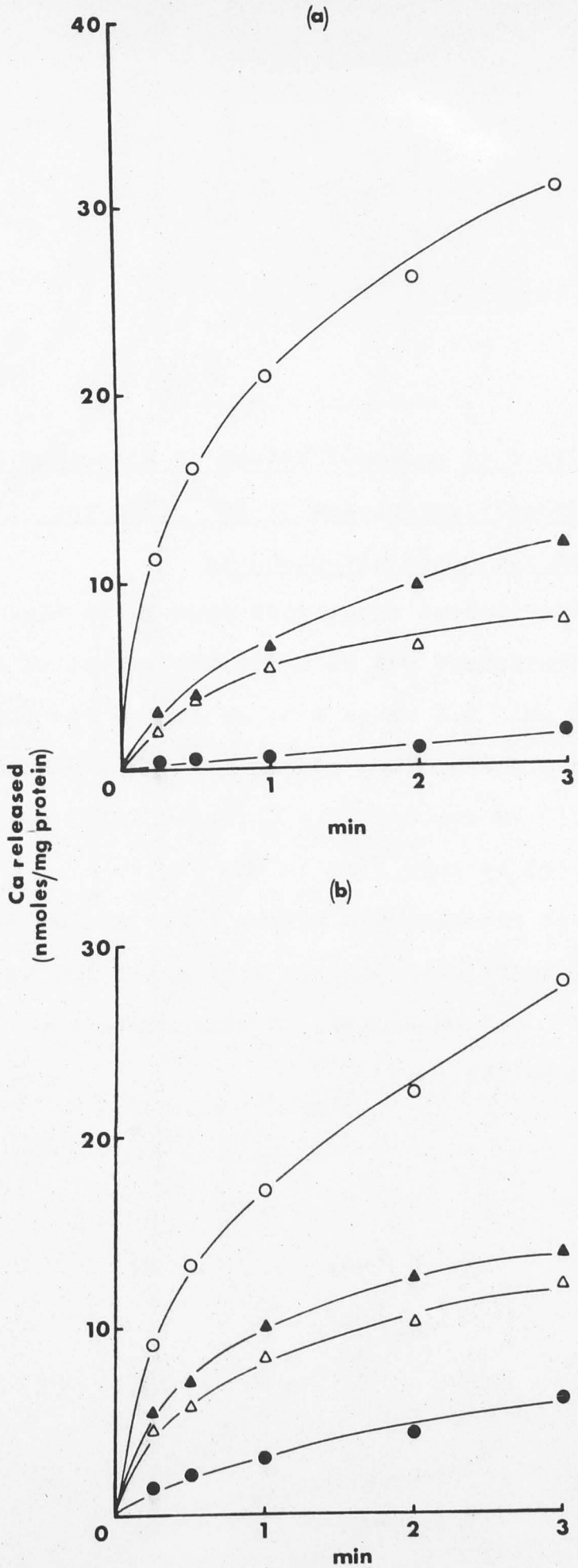

(a)
40
30
20
10
0
1
2
3
min
Ca released
(nmoles/mg protein)
(b)
30
20
10
0
1
2
3
min

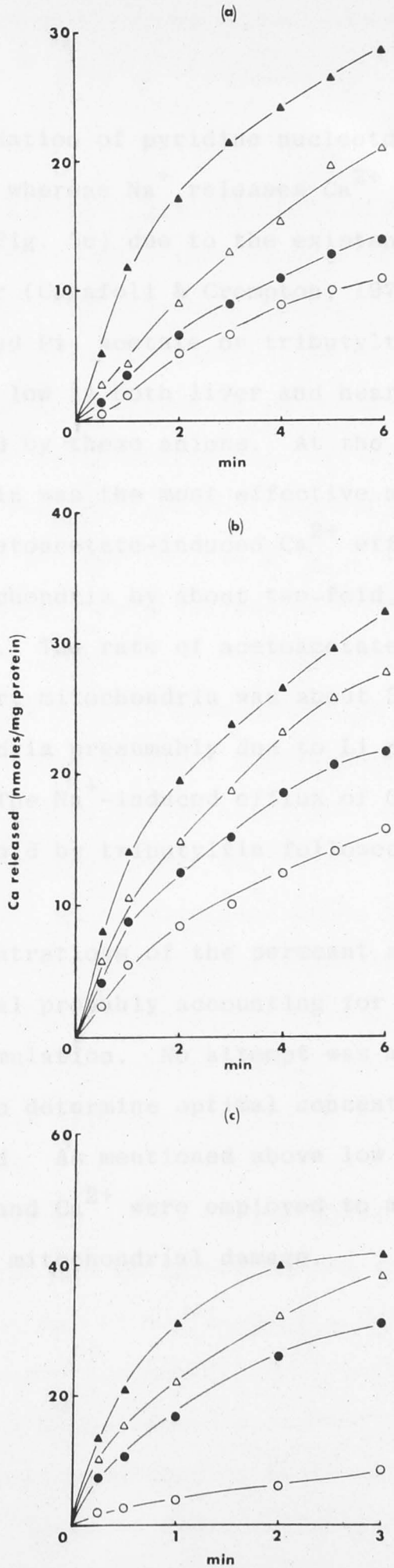
Fig. 5. Effect of permeant anions on Ruthenium Red-insensitive release of Ca^{2+} from rat liver and rat heart mitochondria

The experimental conditions were as in Fig. 4 except that the temperature was 25°C and the amount of added Ca^{2+} was 50 μM. 0.5 μM (a & b) or 1.0 μM (c) Ruthenium Red was added 4 min after adding Ca^{2+}, followed by the addition of 5 mM-acetoacetate (Lithium salt) (a & b) or 20 mM-NaCl (c) at zero time in the Fig.

a - rat liver mitochondria with a LiCl medium was used

b & c - rat heart mitochondria with a KCl medium was used

Control (○), 0.5 mM-Pi (●), 5 mM-acetate (△) and 0.5 μM-tributyltin (▲).


(a)
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Ca released (nmoles/mg protein)
(c)
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due to its oxidation of pyridine nucleotdies (Lehninger et al., 1978b) whereas Na^+ releases Ca^{2+} from heart mitochondria (Fig. 5c) due to the existence of a Na^+/Ca^{2+} antiporter (Carafoli & Crompton, 1978). In the absence of added Pi, acetate or tributyltin, the rate of Ca^{2+} efflux is low in both liver and heart mitochondria and is enhanced by these anions. At the concentrations used tributyltin was the most effective anion in enhancing the rate of acetoacetate-induced Ca^{2+} efflux from liver and heart mitochondria by about two-fold, followed by acetate and Pi. The rate of acetoacetate-induced efflux of Ca^{2+} in heart mitochondria was about 30% higher than liver mitochondria presumably due to Li present in the acetoacetate. The Na^+-induced efflux of Ca^{2+} is enhanced approx. five-fold by tributyltin followed by acetate and then Pi.

The concentrations of the permeant anions employed were not optimal probably accounting for the different degrees of stimulation. No attempt was made in the present work to determine optimal concentrations of the anions required. As mentioned above low concentrations of the anions and Ca^{2+} were employed to minimise secondary effects due to mitochondrial damage.

DISCUSSION

EGTA-induced, Ruthenium Red-inhibited release of Ca^{2+}

The experiments described in this chapter clearly demonstrate that EGTA induces a release of Ca^{2+} from rat liver and rat heart mitochondria; the rate of efflux is dependent on the temperature of the incubation medium and is inhibited by Ruthenium Red. This finding confirms an earlier observation from this laboratory (Reed & Bygrave, 1974b) and from another (Pozzan *et al.*, 1977) using rat liver mitochondria. This inhibition of Ca^{2+} release by Ruthenium Red, the inability of the compound to inhibit efflux of Ca^{2+} induced by the oxidation of pyridine nucleotides in liver mitochondria (Lehninger *et al.*, 1978b) and by Na^{+} in heart mitochondria (Crompton *et al.*, 1976), together suggest that Ca^{2+} could be released from rat liver and rat heart mitochondria by both separate and distinct Ruthenium Red-sensitive and-insensitive pathways (see Table I). The former occurs in response to a lowered cytosolic Ca^{2+} concentration whereas the latter is brought about by specific effectors acting on mitochondria themselves.

The sensitivity of the EGTA-induced release of Ca^{2+} to low concentrations of Ruthenium Red and to temperature suggests that it may be mediated by a specific carrier. Attempts to measure the maximal Ca^{2+}-load inside the mitochondria for EGTA-induced release of Ca^{2+}, failed to detect any substrate saturation (data not shown, see also Harris, 1979). Since no other energy-linked reaction in

TABLE I Ruthenium Red-sensitive and-insensitive release of Ca^{2+} from mitochondria occurring without damage to the organelle

Mitochondrial source	Agent inducing efflux	Inhibited by Ruthenium Red	Other inhibitors
Rat liver	EGTA	Yes[1,2,3]	nupercaine[3]
	pyridine nucleotide oxidation	No[4]	nupercaine[5]
Rat heart	EGTA	Yes[3]	-
	Na^+	No[6]	Lanthanides[7] Bongkrekic acid[8] ADP and ATP[8]
Rat brain	Na^+	No[9]	nupercaine[5]

1. Reed & Bygrave (1974b)
2. Pozzan et al. (1977)
3. present work
4. Lehninger et al. (1978b)
5. Dawson et al. (1979)
6. Crompton et al. (1976)
7. Crompton et al. (1977)
8. Harris (1979)
9. Crompton et al. (1978)

mitochondria so far examined other than those involving Ca^{2+} influx is sensitive to low concentrations of Ruthenium Red (Vasington et al., 1972), we have tentatively identified this release pathway with the Ruthenium Red-sensitive Ca^{2+} influx carrier.

The concentration of Ruthenium Red needed for half-maximal inhibition of EGTA-induced release of Ca^{2+} is about 7 times higher than that needed for inhibition of Ca^{2+} influx (Fig. 2). The reason for the difference in sensitivity to Ruthenium Red is not clear, but the following possibilities may be relevant. First EGTA may bind Ruthenium Red and even only a small degree of binding could markedly reduce the effective concentration of the inhibitor. Second, in the initial rate experiments Ruthenium Red was added before Ca^{2+} accumulation by mitochondria whereas in the efflux experiments the inhibitor was added after the accumulation of Ca^{2+}. The possibility exists that the accumulation of Ca^{2+} by mitochondria induces an alteration in the sensitivity of the Ca^{2+}-carrier to Ruthenium Red. This could result from e.g. a conformational change of the Ca^{2+}-carrier itself (Bygrave et al., 1971; Sandri et al., 1979) or in the inner membrane (Hackenbrock, 1966). The idea that EGTA-induced Ca^{2+} efflux occurs via reversal of the Ca^{2+} influx system is not unique to the mitochondrial inner membrane. Already it has been argued that the release of Ca^{2+} in sarcoplasmic reticulum can occur by reversal of the Ca^{2+} influx transport system (Hasselbach, 1978).

Permeant anions and Ca^{2+} efflux

Proton-donating permeant anions also influenced the Ruthenium Red-sensitive and-insensitive release of Ca^{2+}. The anions tested, Pi, acetate and the Cl^-/OH^- exchange mediated by tributyltin, have been shown to support Ca^{2+} accumulation (Lehninger, 1974; Bygrave et al., 1978b). Ca^{2+} efflux induced by EGTA and which is Ruthenium Red-sensitive, is inhibited by these anions (Fig. 4). However the efflux of Ca^{2+} induced by acetoacetate (Fig. 5a,b) and by Na^+ (Fig. 5c) and which is Ruthenium Red-insensitive, is enhanced by these permeant anions. The reason for the opposite effects mediated by permeant anions on the Ruthenium Red-sensitive and-insensitive efflux of Ca^{2+} is not clear. Both EGTA and Na^+ can enhance the membrane potential, while inducing Ca^{2+} release (Åkerman, 1978a; Nicholls, 1978a), hence the efflux of Ca^{2+} may be electrogenic (Gunter et al., 1978). The permeant anions tested here can restore the Ca^{2+}-induced decrease in membrane potential to its original value without causing any release of Ca^{2+} (see Chapter 5 Fig. 2). Hence the 'strain' induced by the electrogenic pumping of Ca^{2+} against the high membrane potential from the matrix to the medium observed in the presence of these anions is expected to cause an inhibition of Ca^{2+} release. This argument fits well with the efflux of Ca^{2+} induced by EGTA (Fig. 4), but is exactly opposite to the pattern observed when efflux was induced by acetoacetate or Na^+ (Fig. 5).

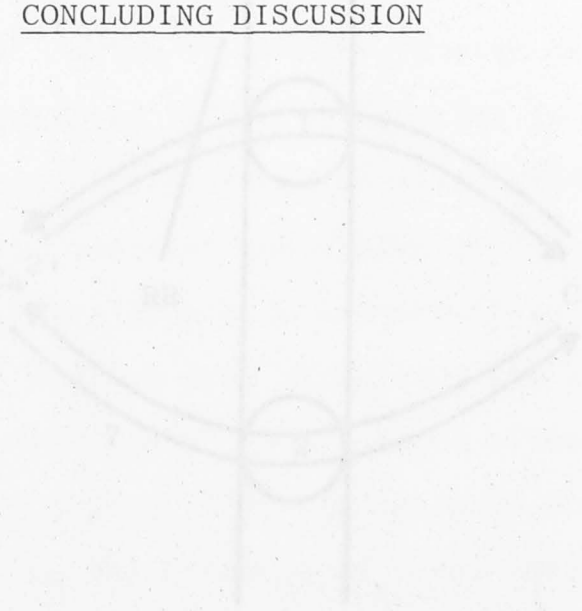
Another possibility independent of the effect of energy is that the pool of Ca^{2+} released by EGTA on the one hand, and acetoacetate and Na^{+} on the other, are different. Two pools which are inaccessible to EGTA can be considered; inner membrane-bound Ca^{2+} and matrix Ca^{2+}. Lehninger (1969) showed that lubrol treatment of mitochondria that had accumulated Ca^{2+}, indicated most of the Ca^{2+} in the absence of Pi or acetate to be membrane-bound whereas in their presence, the ion was located in the matrix. Hence we suggest that Ca^{2+} released in the presence of EGTA arises from inner membrane-bound Ca^{2+} not removable by quenching (Reed & Bygrave, 1974a) whereas the matrix Ca^{2+} is released in the presence of acetoacetate or Na^{+}.

The Ruthenium Red-sensitive and-insensitive release of Ca^{2+} from mitochondria which does not result in damage to the organelle show a number of similarities. These include inhibition by nupercaine (Fig. 3b, Dawson *et al.*, 1979), modification by proton-donating permeant anions (Figs. 4 & 5), sensitivity to pH of the incubation medium, at least in liver (Chapter 6, Fig. 4; Prpic & Bygrave, 1980) and susceptibility to modification by hormone administration *in vivo* in liver (Taylor *et al.*, 1980).

Finally the tributyltin-induced Cl^{-}/OH^{-} exchange is as effective as Pi and acetate not only in the inward movement of Ca^{2+} into mitochondria (Bygrave *et al.*, 1978b), but also in the release of Ca^{2+} from mitochondria, whether or not it is sensitive to Ruthenium Red (Figs. 4 & 5).

CHAPTER 8

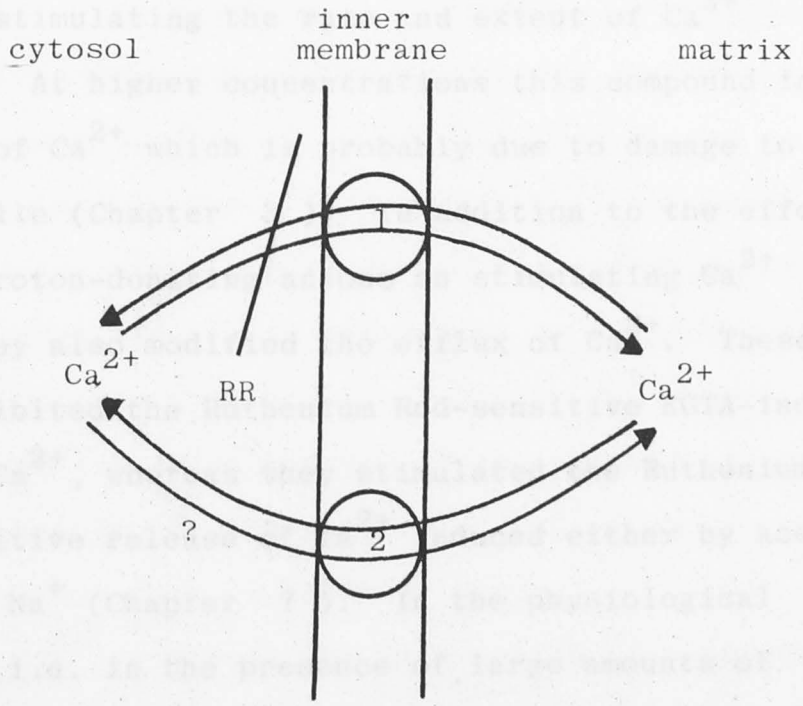
CONCLUDING DISCUSSION



CONCLUDING DISCUSSION

The Ca^{2+}-translocation cycle

As discussed in this thesis, much evidence indicates that the movement of Ca^{2+} across the inner mitochondrial membrane is a bidirectional process. Entry of the ion from the cytosolic side to the matrix occurs via the Ruthenium Red-sensitive carrier whereas the release of the ion from the matrix side to the cytosol occurs through the Ruthenium Red-sensitive or-insensitive carriers (Bygrave, 1978a,b; Carafoli, 1979). This is schematically represented below.



RR - Ruthenium Red

1 - Ruthenium Red-sensitive Ca^{2+} carrier.

2 - Ruthenium Red-insensitive Ca^{2+} carrier.

Schematic representation of the Ca^{2+}-translocation cycle

Role of permeant anions: The work described in this thesis showed that proton-donating permeant anions influence both the influx and efflux components of the Ca^{2+}-translocation cycle (Chapters 3,4 & 7). In the absence of added anions mitochondria have a limited capacity to accumulate Ca^{2+}. Endogeneous Pi movements account for a large fraction of this limited uptake. Hence addition of NEM to inhibit Pi movements decreased this further. In fact, it induced a release of Ca^{2+} that appeared not to be secondary to mitochondrial damage (Chapter 4). It was shown using the anionophore tributyltin, that the Cl^-/OH^- exchange mediated by this compound is as effective as other proton-donating permeant anions in stimulating the rate and extent of Ca^{2+} transport. At higher concentrations this compound induced a release of Ca^{2+} which is probably due to damage to the organelle (Chapter 3). In addition to the effect of these proton-donating anions on stimulating Ca^{2+} influx, they also modified the efflux of Ca^{2+}. These anions inhibited the Ruthenium Red-sensitive EGTA-induced efflux of Ca^{2+}, whereas they stimulated the Ruthenium Red-insensitive release of Ca^{2+} induced either by aceto-acetate or Na^+ (Chapter 7). In the physiological situation, i.e. in the presence of large amounts of cytosolic Pi and HCO_3^-, the Ruthenium Red-insensitive release of Ca^{2+} might take precedence over the Ruthenium Red-sensitive release of the ion.

Effect of pH: The influx and efflux components of the

Ca^{2+}-translocation cycle were shown in this work to be sensitive to pH (Chapter 6). Low pH decreased influx and enhanced efflux, whereas high pH enhanced influx and decreased efflux of Ca^{2+}. The sensitivity of the mitochondrial Ca^{2+}-cycling to pH appeared not to be secondary to changes in the mitochondrial energy status. It is not known whether the changes in response of the mitochondrial Ca^{2+} transport system to medium pH is of any physiological significance.

It is worth noting however that mitochondria isolated following administration of glucagon to rats (Prpic et al., 1978) or perfusion of livers either with glucagon or α-adrenergic agonists (Taylor et al., 1980), show an enhanced Ca^{2+} retention in vitro and increased transmembrane pH gradient. The increase in the pH gradient of about 0.15 pH units may be one of the factors responsible for mitochondrial Ca^{2+} retention.

One of the early events during fertilization of sea urchin eggs also is an alteration in cytosolic pH as well as mobilization of intracellular Ca^{2+} (Epel, 1977). It is not known however if the altered cytosolic pH is responsible for the release of Ca^{2+} from intracellular sources that is known to occur.

Cycling of Ca^{2+} in vivo: The interaction of α-adrenergic agonists with rat liver leads to a cAMP-independent, Ca^{2+}-dependent activation of phosphorylase (Assimacopoulos-Jeannet et al., 1977; Keppens et al., 1977; Blackmore et al., 1978). This stimulation is accompanied by a large

mobilization of intracellular Ca^{2+} (Blackmore et al., 1978; Chen et al., 1978), which is postulated to activate phosphorylase b kinase, thereby enhancing glycogenolysis. The mobilized Ca^{2+} is expelled from the cell (Blackmore et al., 1978; Chen et al., 1978). One of the major intracellular sources for Ca^{2+} has been shown to be the Ca^{2+} pool located in the mitochondria (Babcock et al., 1979; Blackmore et al., 1979). In addition, glucagon, vasopressin and angiotensin II also cause release of Ca^{2+} from liver cells (Chen et al., 1978; Blackmore et al., 1979), derived partly from mitochondria (Blackmore et al., 1979). The α-agonists, vasopressin and angiotensin II can cause as much as 50 to 70% loss of mitochondrial Ca^{2+}, whereas that induced by glucagon is only about 10 to 20% (Blackmore et al., 1979). However within a short time the cellular Ca^{2+} content (Blackmore et al., 1979) as well as that of the mitochondria (Taylor et al., 1980) return to its original (pre-hormone treated) level. This movement of mitochondrial Ca^{2+} provides strong evidence for the operation of a Ca^{2+}-translocation cycle across the inner membrane in vivo. It also suggests that this cyclic movement of Ca^{2+} may be a target for hormone in mediating some of their effects.

Protonmotive force and mitochondrial Ca^{2+} transport

The ion distribution technique of Nicholls (1974) was used in the present study to follow the components of the protonmotive force. The limitation of this technique restricts quantitative correlations from being made.

This arises mainly due to the inability to correct for any binding of the inhibitors used as well as due to poor time resolution. The latter problem does not arise in steady-state measurements. One of the major requirements in initial rate studies is for an automated technique permitting measurement of membrane potential and Ca^{2+} transport. A study using the safranine technique for measuring membrane potential and the arsenazo or similar dye technique for Ca^{2+} transport, with a stopped-flow apparatus will be most useful in the future.

Nevertheless the data presented in this thesis show clearly that Ca^{2+} transport induced a decrease in membrane potential and an increase in pH gradient (Chapter 5). This finding provides direct evidence for the electrophoretic nature of Ca^{2+} transport (Selwyn et al., 1970a; Lehninger, 1974). The entry of Ca^{2+} into mitochondria is inherently fully charge-compensated by the ejection of about two H^{+} for each Ca^{2+}. Hence Ca^{2+} enters the mitochondria through a uniporter (see Chapter 1 P 20). The interaction of Ca^{2+} with mitochondria is very rapid with a half-time of about 70 msec (Chance, 1965). Hence it is not possible with present techniques to determine whether the decrease in membrane potential or the increase in pH gradient occurs first. But it is logical to assume that the electrophoretic movement of Ca^{2+} causes initially a decrease in membrane potential which then results in a stoichiometric stimulation of respiration by the mitochondria (Rossi & Lehninger, 1964) and it is this, that is responsible for the enhanced

pH gradient.

The initial rate of Ca^{2+} transport appears not to be limited by the driving force, the membrane potential. In chapter 5 it was shown that smaller changes in the membrane potential caused larger changes in Ca^{2+} transport. In chapter 6 it was shown that in the presence of Pi, the initial rate of Ca^{2+} transport changes markedly as a function of the pH of the incubation medium despite the maintanance of a similar membrane potential. Experiments carried out in collaboration with T.P. Heaney showed that various mitochondrial fractions (heavier fractions), had identical membrane potentials, transmembrane pH gradients, state 4 and state 3 rates of respiration and cytochrome oxidase activity, but altered Ca^{2+} transport, which was stimulated to varying degrees by Pi (Bygrave et al., 1978a). Moreover changes in mitochondrial Ca^{2+} transport in tumour cells (Bygrave, 1976), and in response to hormones (Dorman et al., 1975), to diet (Bygrave & Smith, 1978) and to development (Bygrave et al., 1975; Smith & Bygrave, 1978) appear not to be due to alterations in the energy status of the organelle.

On the other hand Heaton & Nicholls (1976) and Hutson (1977) showed using the Ca^{2+} ionophore A23187 that the activity of the Ca^{2+} transport system in the steady-state is limited by the activity of the respiratory chain.

Mitochondrial enzymes like pyruvate dehydrogenase (Denton et al., 1975), 2-oxo glutarate dehydrogenase

(McCormack & Denton, 1979) and NAD-linked isocitrate dehydrogenase (Denton et al., 1978) are activated by Ca^{2+} in vitro with maximal effects at about 1 μM-free Ca^{2+}. The concentration of ionic Ca^{2+} in mitochondria can be calculated using the Nernst equation, knowing the membrane potential and the ionic Ca^{2+} concentration in the medium under equilibrium conditions. However the distribution of Ca^{2+} in the steady-state appears not to be in equilibrium with the membrane potential presumably due to the existence of efflux pathways (Puskin et al., 1976; Azzone et al., 1977; Pozzan et al., 1977). The only alternative available now to measure free Ca^{2+} in the mitochondria is to use Mn^{2+} as a paramagnetic analogue for Ca^{2+}. These studies reveal that free Mn^{2+} concentration in mitochondria in vitro can be as high as 40 mM (Puskin et al., 1976). Large amounts of Mn^{2+} are bound internally depending on the pH of the matrix (Puskin et al., 1976). The total concentration of Ca^{2+} in freshly-isolated mitochondria is of the order of 10-20 mM (Lehninger et al., 1967). Ca^{2+}, among physiologically-important divalent cations appears to have an irregular coordination geometry in model complexes (Williams, 1976). Hence a large amount of Ca^{2+} must be bound, by analogy with Mn^{2+}. The determination of the ionic concentration of Ca^{2+} in the matrix is another important problem to be faced.

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